Aus dem Institut für Phytopathologie
der Christian-Albrechts-Universität zu Kiel

Establishment and persistence of the entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*

Dissertation
zur Erlangung des Doktorgrades
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vorgelegt von

M. Sc. Ismail Alper SUSURLUK

aus Trabzon, Türkei

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Dekan:	Prof. Dr. S. Woffram
Erster Berichterstatter:	PD. Dr. habil. R.-U. Ehlers
Zweiter Berichterstatter:	PD. Dr. habil. J. Aumann
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„Hayatta en hakiki mürsit; ilimdir, fendir“

Mustafa Kemal Atatürk

„Es ist nicht genug, zu wissen, man muss auch anwenden;
es ist nicht genug, zu wollen, man muss auch tun“

Goethe, Johann Wolfgang

„There are no shortcuts in the quest for perfection“

Ben Hogan

Table of Content **Pages**

1. Introduction

The development of resistance to chemical insecticides is one of the driving forces for change in insect pest management. At present, the increasing number of resistant insects is outstripping the development of new insecticides. The number of registrations of new compounds is slowing down due to regulatory policy. Governments today demand environmentally safe chemicals with low toxicity and short-term persistence, low mobility to avoid ground water contamination and limited effects on non-target organisms. These prerequisites have reduced the chances of finding new, effective insecticides and have dramatically increased the costs for the development of new compounds, especially costs related to toxicology and safety research (Ehlers, 1996). For the chemical control of soil-borne insect pests, this situation is without remedy and is augmented by reports of failures with traditional soil insecticides (e.g. Racke and Coats, 1990). In comparison with the phylloplane, the conditions in soil present major problems for chemical control. Insects often occur deep in the soil. To reach them, a chemical compound must leach with the soil water, which increases the probability of groundwater contamination. Mineral and organic soil particles are of infinite surface area and agricultural soils contain a diversity of micro-organisms. Due to absorption to soil particles and/or biodegradation by soil organisms, successful control can only be achieved by using highly toxic and long term persistent chemicals. Compounds with these characteristics will hardly meet regulatory requirements. Moreover, residues of insecticides have a negative effect on the environment as well. Many insecticides kill natural enemies of insects. Consequently, the conditions set for the development of effective soil insecticides contradict the objectives of modern regulatory policy. The registration of new chemicals for the control of soil insects is improbable, as the requirements have become almost insuperable. Therefore, the development and implement of alternative control strategies are needed. One important alternative control measure providing environmentally friendly and sustainable plant protection is biological control. Insect control can no longer rely on insecticides alone, and the significance of biological control agents as part of integrated pest management programmes will increase continuously in the future. Biological control offers a tremendous opportunity to supply agriculture with effective tools for the development of production techniques, which minimise impacts on human health and the environment (Ehlers, 1996). The success of biological control, however, will also depend on the understanding of adaptation and establishment of introduced biological control agents in agricultural ecosystems.

Entomopathogenic nematodes: Besides microbial pathogens and arthropod biocontrol agents of pest insects, entomopathogenic nematodes (EPNs) have been successfully used in agricultural systems. Like parasitoids or predators, EPNs have chemoreceptors and are motile. Like pathogens, they are highly virulent, killing their hosts quickly and they can be cultured easily *in vivo* or *in vitro* (Ehlers, 2001). In addition to above attributes, they are safe to most non-target organisms and to the environment. No evidence exists for a mammalian pathogenicity (Ehlers and Peters, 1995; Boemare et al., 1996; Ehlers and Hokkanen, 1996). There are also no difficulties to apply EPNs as they are easily sprayed using standard equipment and can be combined with almost all chemical control compounds (Georgis and Mamweiler, 1994; Georgis and Kaya, 1998). EPNs have been used against many different pests in the soil, in cryptic habitats and on the foliage (Begley, 1990). Soil is the natural reservoir of EPNs (Akhurst, 1986; Gaugler, 1988) offering excellent conditions for nematode survival and activity. The opportunity to use EPN is promising more than 90% of insect pests spend part of their life cycle in the soil. The use of EPNs for biocontrol began only in the early 1980s and involved a step-by-step scientific and technical development. Mass production of the nematodes played a key role in the commercially development of insect pests control with nematodes (Ehlers, 2001).

EPNs are known since 1923 when Steiner identified the species, *Aplectana kraussei*. Then, Glaser and Fox (1930) found a nematode infecting grubs of the Japanese beetle, *Popillia japonica* at the Tavistock Golf Course near Haddonfield, New Jersey, USA. This nematode was described by Steiner as *Neoplactana* (=*Steinernema*) *glaseri* (Steiner, 1929). Later, Glaser and his colleagues propagated sufficient amounts of the species for field trials. The species was applied in 1930s in 73 different field plots to control *Popillia japonica*. A new genus *Heterorhabditis* was described by Poinar (1975) in the family Heterorhabditidae. The family is very similar with the family Steinernematidae. Currently, 48 species of EPNs have been described from all over the world (Hominick, 2002).

Biology and life cycles of the entomopathogenic nematodes: EPNs that belong to both genera (*Heterorhabditis* and *Steinernema*) have several developmental stages, which are eggs, four juvenile stages (J1-J4) and the adult stage. Their life cycle consists of a free-living phase of the infective juvenile (IJ), also called dauer juvenile (DJ), occurring in the soil and the propagative phase, which occurs inside of the insect host body. The term dauer (German word for enduring) was introduced by Fuch (1915). The IJ stage is the 3rd stage juvenile and it is

morphologically and physiologically adapted for long-term survival and formed as a response to depleting food resource and adverse environmental conditions in the soil. The IJs are morphologically distinct from propagative J3 stages (Johnigk and Ehlers, 1999). Due to their ability to survive without food they can be used for insect control purposes. While moving through the soil, IJs of genus *Steinernema* loose their J2 cuticle; IJs of the genus *Heterorhabditis* retain the J2 cuticle until they enter a host. IJs can survive extended periods in the soil while waiting for the opportunity to infect a host. The IJs of *Steinernema* and *Heterorhabditis* species are symbiotically associated with bacteria of the genera *Xenorhabdus* and the bioluminescent *Photorhabdus*, respectively. Symbiotic bacteria of both genera are motile and gram-negative, and belong to the family Enterobacteriaceae, within the gamma subdivision of the purple bacteria (Ehlers et al. 1988; Forst and Clarke, 2002). An IJ carries between 0 and 2000 cells of its symbiont bacterium in the anterior part of the intestine (Spiridonov et al., 1991; Sturhan and Kreimeier, 1992; Endo and Nickle, 1994). *Xenorhabdus* occurs naturally in a special intestinal vesicle of *Steinernema* IJs (Bird and Akhurst, 1983), while *Photorhabdus* is distributed in the foregut and midgut of *Heterorhabditis* IJs (Boemare et al., 1996). Typical for the symbionts of both genera is the phenomenon of phase variation, two extremes that are the primer and the secondary phase (Akhurst, 1980). Intermediate phases have been reported (Gerritsen and Smits, 1997). The primary phase is isolated from the IJ or infected insects, whereas the secondary phase occurs either after in vitro subculturing or in vivo, when the EPN emigrate from the cadaver (Grunder, 1997). Some strains of *Xenorhabdus* and *Photorhabdus* are highly virulent. Infection with less than 10 bacterial cells of the bacteria into the host insect haemocoel can be sufficient to kill a susceptible insect such as *Galleria mellonella* or *Manduca sexta* L. (Forst et al., 1997; Ffrench Constant and Bowen, 2000). EPNs develop and reproduce in the insect and feed on the symbiotic biomass provided by the bacteria.

Upon location of a potential host, the IJs move towards the host and penetrate into the insect body via the mouth, anus, spiracles, or direct penetration through the cuticle. Once in the insect cavity, symbiotic bacteria are released from the nematode gut, which multiply rapidly in the haemolymph and causes insect death within 48 hours after penetration (Götz et al., 1981). The life cycle of *Heterorhabditis* is similar to that of steinernematids excepting for the fact that the IJs always develop into self-reproducing hermaphrodites (Poinar, 1990). Strauch et al. (1994) observed that offspring of the first generation hermaphrodites can either develop into amphimictic adults or into automictic hermaphrodite; both can occur simultaneously (Fig.

1 and 2). The development into amphimictic adults is induced by favourable nutritional conditions, whereas the development of hermaphrodites is induced by low concentrations of nutrient. The life cycle is completed in a few days and thousands of new IJs emerge in searching for new hosts. The cycle from entry of IJs into a host until emergence of new IJs is dependent on temperature and varies for different species and strains. It generally takes about 6-18 days at temperatures ranging from 18 to 28 °C in *G. mellonella* (Poinar, 1990; Nguyen and Smart, 1992).

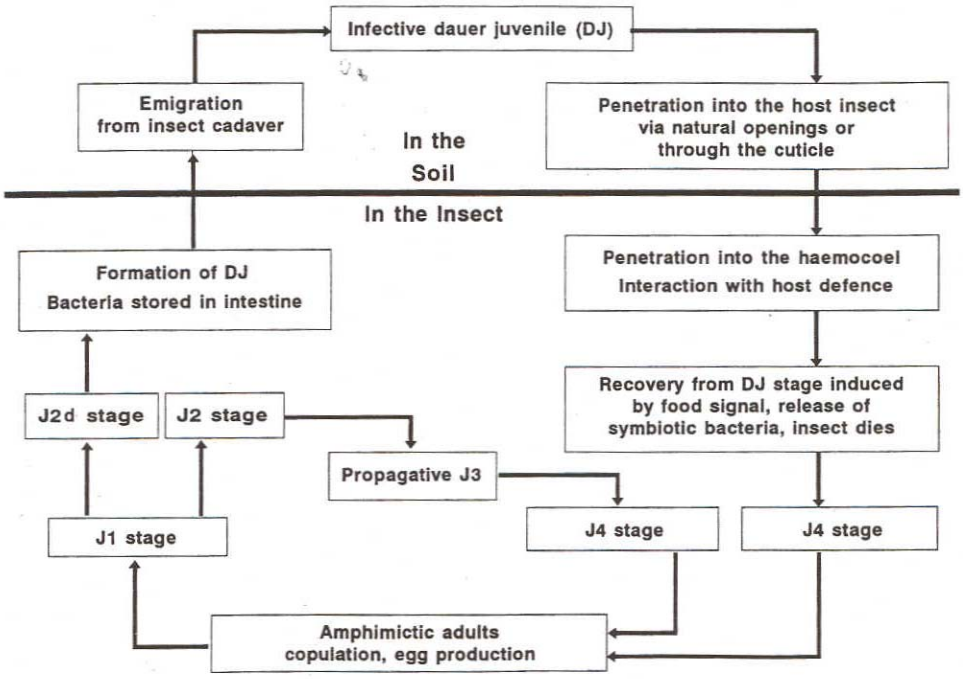

Figure 1. Life cycle of *Steinernema* spp. (J1-J4: Juvenile stages; J2d: Pre-dauer juvenile) (Ehlers, 1996)

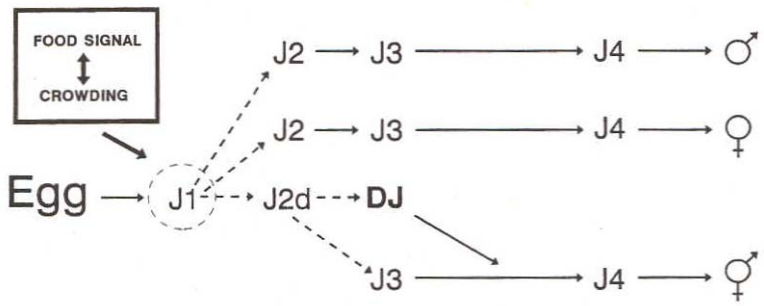

Figure 2. Alternative development pathway of *Heterorhabditis* spp. either to amphimictic females and males or via the pre-dauer stage (J2d) to automictic IJs and hermaphrodites (Strauch et al., 1994).

Persistence of entomopathogenic nematodes: Efficacy of applied EPNs relates closely to the used strains or species and environmental and technical conditions, that no two situations are identical or comparable. Factors of major importance are age and lipid reserves of IJs. These characteristics directly influence the ability of nematodes to survive a period of time without a host and their ability to find and infect a host (Womersley, 1993). Many studies mainly in the laboratories have tested the persistence of EPNs under various conditions in sterile soil. The data generally indicated a survival of weeks rather than months and a gradual decline in the numbers of living nematodes recovered. In some cases, however their infectivity potential, however, does not follow the same pattern because at least some nematode individuals are capable of entering a quiescent state and later become active again when conditions permit host-finding activity (Fan and Hominick, 1991; Womersley, 1993). Behavioural adaptations and phases of anhydrobiosis or quiescence (Womersley, 1993) will influence the pattern of persistence. Nematodes developed strategies to survive adverse environmental conditions. In a dormant stage, the quiescent (Glazer, 2002) their metabolism is lowered and a longer persistence is possible. However, nematodes in this condition are not pathogen and only if the environmental condition are favourable, they retain active and are able to penetrate into insects. The quiescent stage can be induced by extreme temperatures, oxygen deficiency and high salt content (Glazer, 2002).

After application nematodes rapidly disappear (Molyneux, 1985; Kung et al., 1991). But after the rate of population decline slows down. Several factors such as soil type (Kung et al., 1990a), humidity levels in soil (Kung et al., 1991), temperatures (Griffin, 1993; Grewal et al., 1994) and soil pH (Kung et al., 1990b) affect the persistence and infectivity in soil. The temperature tolerance of the IJs depends on their geographical origin. Extreme temperatures are generally unfavourable for the persistence (Kaya, 1990). Nematodes with origin from warmer areas survive better at higher temperatures, while nematodes with origin from cooler regions survive better at cooler temperatures (Kaya, 1990; Curran, 1993; Griffin, 1993).

The long-term persistence of EPN population depends on their ability to find and infect host and produce offspring. In addition to abiotic factors, some biotic factors in the soil have influence on their persistence abilities. Biotic factors are natural enemies and intraspesific or interspecific competition and the presence of host insects. Kaya (1990) identified the major natural enemies as collembola, mites, nematophagous fungi, predatory nematodes and

microsprodia. Ishibashi and Kondo (1987) pointed out that nematodes have higher survival rates in sterilised soil than in unsterilised soil.

Very little information is available on the natural hosts and host specificity of EPNs in nature. Our understanding of EPN population dynamics over the seasons and their possible synchronisation with host life cycles is also very limited (Smits, 1996). Nowadays, nearly all investigations about persistence have shown a strong relationship with the soil environments. No information is available about successful establishment EPNs and the increases their persistence in agriculture ecosystems. In order to achieve sustainable effects with introduced EPNs, the successful establishment and subsequent persistence in the agro-environment is of major importance.

Objectives of the thesis: Two major strategies exist in biological control. Inundative release of biocontrol agents follows the "bioinsecticide" strategy. The control agent is applied when problems occur. Classical control introduces antagonists to achieve long-term effects. This approach is often taken to regulate accidentally introduced pests. Today, EPN are only used inundatively and the use is limited to high value crops like ornamentals, mushrooms, vegetables and orchards (Grewal, Ehlers and Shapiro, in press). Little is known about the impact of natural occurring EPN populations (Ehlers, 1998). Reports can be divided into relatively balanced host-nematode associations and epizootics. A more balanced relation was reported for *S. kraussei* and/or *S. feltiae* with the forest pest spruce webworm *Cephalcia abietis* (Hymenoptera) with a cumulative annual control of 25% (Mracek, 1986; Eichhorn, 1988). Epizootics have been only reported with *Heterorhabditis* spp. reaching 71% control in a sugarcane grub pest population (Akhurst et al., 1992).

One major reason why nematodes are only used in high value crops is the relatively high costs related with the use of EPN. For instance, the application against the cabbage root fly *Delia radicum* (Diptera: Anthonomyiidae) in oil seed rape would cost approximately 900 €/ha. Although product costs are declining in the future (Ehlers, 2001), they are still too high to consider the use of EPN in agriculture environments. However, reports on long-term effects encourage the research to investigate the potential of EPN as antagonists also in large-scale agriculture ecosystems. For example, if an establishment of *S. feltiae* in a field, which is in the three year rotation wheat-barley-oil seed rape, have a stabilizing effect on the population of

the cabbage root fly, the one time application, maybe even at lower densities, would be cost effective.

If this is attempted then information will be necessary to establish nematodes in agriculture ecosystems and, if long-term effects are part of this strategy, information on the persistence must be available.

Establishment of EPN was investigated in different outdoor crops by spraying *H. bacteriophora* and assessing the influence of the time of application (March until July) and the crop (e.g. wheat, field beans, meadow). Strawberries plastic mulch is often used to control weeds. Then EPN cannot easily be applied to the soil surface. Therefore, dipping of plant prior to planting was investigated as a potential method for establishment.

Persistence was investigated with *H. bacteriophora* and *S. feltiae* after spraying in different field crops. The tests were carried out in conventional and organic farming systems between 2001 and 2004. Persistence of *H. bacteriophora* was assessed also in strawberries after dipping of roots and the effect on the major strawberry pest, the Black Vine Weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) was recorded.

H. bacteriophora is not commonly found in Germany. Sturhan (1996) reports only three records and Ehlers (personal communication) has encountered this species only two times. Thus it can be considered as an exotic species at the area of release. Although no major negative impacts of EPN on the environment were reported (Ehlers and Hokkanen, 1996), the question was raised by Barbarcheck and Millar (2000) whether introduced species can replace indigenous species. In this study, we therefore recorded the occurrence of indigenous EPN species was therefore recorded before and after the release of *H. bacteriophora* and EPN isolates were identified by molecular analysis (PCR-RFLP) following the methods described by Reid and Hominick (1998).

There is little or no understanding of the population dynamics of applied EPNs over the seasons and of their possible synchronisation with host insect and/or crop in fields. Such information is required to understand persistence and establishment of EPNs in agro-ecosystems. Persistence is influenced by the crop and host insect. The long-term persistence of EPN population depends on their ability to find and infect hosts and to produce offspring.

After several months, it can be questioned whether their infectivity is sufficient to enable them entering and killing a host or not. The availably of suitable hosts every few months seems to be a prerequisite for EPN populations to recycle and persist over longer period. Therefore the occurrence of potential host insects was also recorded. *Sitona lineatus* L. (Coleoptera: Curculionidae) and *Delia radicum* Bouche (Diptera: Anthomyiidae) were frequently detected and therefore their potential to serve as hosts for the nematodes were investigated. In these experiments data on the susceptibility of the insects as well as the reproduction potential of the nematodes in these hosts were assessed.

In order to establish and persist, nematodes must be able to find their potential hosts. To get information on the host finding ability of *S. feltiae*, their reaction to the pest *D. radicum* and the plant oil-seed rape was monitored in Y-olfactometer apparatus at two different temperatures.

Once nematodes are released it might be of interest whether a population which has been propagated in 3,000 l bioreactors over several generations will be genetically stable or adapt to the field condition. After one year nematodes recovered from the fields, they were therefore examined if they had maintained their control potential and capability for reproduction, pathogenicity and persistence.

2. Material and Methods

2.1. Media and Chemicals

- ***Galleria mellonella*- medium (Wiesner, 1993)**

Corn groats (polenta)	22%
Wheat flour (full com)	22%
Milk powder (skim-milk)	11%
Honey	11%
Glycerol	11%
Yeast powder (Brewer`s beer yeast)	5.5%
Bee-wax	17.5%

- **Ringer´s solution (Laboratory standard)**

NaCl	9 g
KCl	0.42 g
$CaCl_2$ x $2H_2O$	0.37 g
$NaHCO_3$	0.2 g
Aqua dest.	1,000 ml

- **YS-medium (Dye, 1968)**

Yeast extract (Merck)	5.0 g
NaCl	5.0 g
$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4$ x $7H_2O$	0.2 g
Aqua dest.	1,000 ml

2.2. Organisms

2.2.1. Insects

***Galleria mellonella**:* The grater wax moth larva *Galleria mellonella* L. (Lepidoptera: Galleridae) was used for nematode baiting and produce progeny of nematode isolates. The insect culture was reared in 1,500 ml volume glass containers (11 cm diameter and 15 cm height) at 30-32 °C on an artificial medium according to Wiesner (1993). The glass containers were closed with filter paper and a metal screen. Females laid eggs on the filter paper from where they were collected and transferred into fresh medium. The eggs hatched within 3-4

days. Larvae were fed weekly. After 5-6 weeks, larvae reached the last instar and were collected to be used in the experiments. Some larvae were left in the containers to pupate. Two weeks later, the adult females emerged and laid eggs.

Preparation of the artificial medium: Mix honey, glycerol and yeast-powder at 80 °C until mixture is homogenous. Melt bee-wax separately at 80 °C. Add cereals and milk powder to the honey-glycerol-yeast mixture, then add melted bee-wax and mix ingredients, until medium is almost homogenous.

Otiorhynchus sulcatus: The larvae of the Black Vine Weevil *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae) were used in pot experiments to test efficacy and persistence of *H. bacteriophora* and application by dipping of roots. The adults of the insect were collected from park areas and strawberry fields in the federal state of Schleswig-Holstein (Germany) and were also received from Dr. Reinhard Sol (Reinbek, Hamburg). The adult weevils were reared on yew leaves, *Taxus baccata* L., in glass jars at a temperature of approximately 20-25 °C (Boff et al., 2002; van Tol, et al., 2001). The eggs collected from the jars were transferred to strawberry plants, *Fragaria vesca* L. planted in pots. The young strawberry plants (cold stored "Frigo" plant) were supplied by the company Kraege Beerenobst Spezialkulturen (Delsener Heide, Telgte, Germany).

Delia radicum: The cabbage root fly larvae, *Delia radicum* Bouche (Diptera: Anthomyiidae) were collected from oil-seed rape fields in Rastorfer Passau (Kreis Plön, Germany) and were reared on the oil-seed rape plants, *Brassica napus* var. *napus* L., which had been transferred from the same field to 20 °C and reared according to the method described by Harris and Svec (1966).

Tenebrio molitor: The mealworm larvae, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were used to determine the occurrence and pathogenicity of *H. bacteriophora* in pot experiments. The larvae were supplied by the company Morio Zoobedarf (Röttingen, Germany).

2.2.2. Entomopathogenic Nematodes

Entomopathogenic nematodes, *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae) strain en02 (Nemaplus®) and *Heterorhabditis bacteriophora* Poinar (Rhabditida:

Heterorhabditidae) strain en1 (Nematop®) were used in the experiments to investigate establishment and persistence. The nematodes were supplied by the company e-nema GmbH (Raisdorf, Germany) and had been cultured according to Ehlers et al. (1998; 2000) and Ehlers (2001).

2.3. General Methods

2.3.1. Nematode *in vivo* culture

To culture nematodes isolated from the field, they were propagated on *G. mellonella* according to Kaya and Stock (1997). They were harvested with water traps consists of a cylindrical plastic container (9 cm height x 8 cm diameter) containing a 5 cm diameter inverted Petri dish placed at the bottom and filled with Ringer´s solution. On the top of the inverted dish a filter paper (Whatman # 1.9 cm diameter) was placed with its edges in contact with Ringer´s solution. Dead insect larvae were placed on top of the filter paper. After 10-15 days the IJs emerged from the insect cadaver migrated over the filter paper and ended up in the Ringer´s solution from where they were collected.

2.3.2. Control of EPN survival after spraying

To evaluate the effect of the spaying on survival of EPN, 5 samples of 100 µl were taken from the spraying tank and the number of IJs was counted under the microscope. This procedure was repeated with nematodes, which were sampled in Petri dishes after spraying (2.3.4).

2.3.3. Control of fitness before and after spraying

To compare the fitness of EPN before and after spraying, nematode performance was tested in a bioassay with *G. mellonella*. Nine cm diameter Petri dishes were filled with moist silver sand (10 % w/v) of 0.1-0.5 mm particle size, which had been sterilised at 80°C for 12 h. Five last instar *G. mellonella* were transferred into the dish and 100 IJs per insect were applied. The insects were incubated for 5 days at 25°C and then the mortality was assessed. The experiments were always done in 10 replicates.

2.3.4. Quality control assay

A general quality control was performed with nematodes applied in fields in 2004. The pathogenicity of *H. bacteriophora* was determined by evaluating the LD_{50} against *T. molitor* larvae. The experiments were conducted in boxes (13 length x 13 width x 5cm height, 500 ml volume) filled with moist sand (as described in the prior assay). Twenty *T. molitor* larvae were added per box. Four different nematode dosages (5, 10, 20 and 30 IJs per larvae) were applied to the boxes. Each dosage was replicated 10 times. Mortality was assessed after 5 days.

2.3.5. Bioassay to test the infectivity at different temperatures

Concentrations of 5, 10, 20, 30, 60 and 120 IJs/*T. molitor* larvae were tested at temperatures of 12, 18 and 24 °C. The bioassay was carried out in small boxes (5 x 5 x 5 cm) filled with moist silver sand (as previously described) and 10 last instars of *T. molitor*. After application of *H. bacteriophora*, the boxes were incubated at above described temperatures. The experiment was replicated 10 times. After 5 days mortality of the insects was assessed.

2.3.6. Assessment of *in vivo* reproduction potential

In order to evaluate whether the reproductive potential of EPN was affected, the number of nematode offspring per insect was evaluated. Experiments were carried out as described in the infectivity bioassays using the concentrations of 10, 50 and 100 IJs per *G. mellonella* larva. In this assay, *G. mellonella* larvae were used with a weight between 145 and 188 mg. Each concentration of the IJs was tested on 10 *G. mellonella* larvae. After 48 h incubation the infected cadavers, recognised their red colour, were removed from the sand, rinsed, transferred to water traps (2.3.1.) and incubated in the dark at 25 °C. All emerging IJs from a single host insect were recovered over a period of 10 days and stored in a 50 ml flask (Boff et al., 2000). The content of each flask (nematode suspension from individual cadavers) was mixed thoroughly using air bubbles. Eight samples of 10 µl from each suspension were examined under a stereomicroscope and the total number of IJs per cadaver was calculated.

2.3.7. EPN application

In field trials, nematodes were sprayed using a concentration of 5 x 10^5 IJs m^{-2}. For each treatment 120 million IJs were applied with 10 l water on each plot of 240 m² (~ 41.5 ml water m^{-2}) with an experimental plot application system (Pneumatic Sprayer Type PSG, Schachtner, Ludwigsburg, Germany). The sprayer was equipped with two spray tanks with a volume of 2.5 and 7.5 l, a 5 litre-volume pressure tank and an electronic tachometer. The sprayer was operated at a pressure range of 1.5 – 6.0 bars. Two rods of each 1.5 m are equipped with three spraying nozzles (0.5 mm diameter) in distance of 50 cm (Figure 1). The applications were performed using 4-5 bar pressure at a velocity of 1 m s^{-1}. Applications were conducted on plots of 240 m² (80 m length x 3 m width). The distance between the experimental plots was at least 10 m. The sprayer was moved forward and backwards over the plot to treat each plot two times. The nozzle height was kept constant at 50 cm distance from the top of the plants. Prior to the applications, tap water and EPN formulation were gently mixed in a plastic barrel and then the EPN solution was transferred into the spraying tanks. To prevent sedimentation of the nematodes in the tank, which would result in a non-homogenous distribution in the field, the tanks were well shaken before application. During the application the number of EPN cm^{-2} was assessed by placing Petri dishes in the field and the number of nematodes was later counted in the laboratory (2.3.4.)

To assess droplet size and number of IJs per drop specific for the used spraying equipment, the sprayed drops were once sampled in Petri dishes and their size was measured using the software program, Analysis® (Soft Imaging System, Münster, Germany). The amount of drops reaching the soil and the number of IJs per drop were evaluated.

For spraying of EPN in laboratory experiments a 500 ml volume hand sprayer was used, which was equipped with a 0.5 mm diameter nozzle.

Figure 3. Experimental plot sprayer Schachtner Type PSG.

2.3.8. Quantification of application density

In order to obtain the number of applied IJs per area immediately after application, 10 plastic Petri dishes (9 cm diameter) were put in 10 m intervals onto the soil surface in the experimental plots. After application, the Petri dishes were collected and the nematode suspensions were rinsed into plastic tubes with Ringer´s solution. In the laboratory, the number of IJs per tube was counted to calculate the amount of IJs applied per cm² soil. This method was conducted during each field application.

2.3.9. Soil sampling

Soil samples were collected prior to and after EPN application. Each sample contained approximately 40 g soil from an area of 3 cm². The samples were placed in a plastic bag and transported to the laboratory. The samples were kept at 4 °C until analysed.

2.3.10. Assessment of establishment and persistence

In order to assess establishment after application and persistence over time, soil samples were taken in the field from treated and from control plots and the occurrence of EPN was evaluated. The total number of nematodes was not assessed, but the percentage of soil samples in which EPN were detected. Detection was done by the *G. mellonella* baiting method according to Bedding and Akhurst (1975). Soil was homogenised by hand and each soil sample was placed in a 90 ml plastic box (5 x 5 x 5 cm) with two last instar larvae of *G.*

mellonella. After the larvae had been added, the boxes were turned round and stored at 25 ± 2 °C. Three days later, dead larvae were examined for nematode infection. Larvae with a red colour were recorded as positive for *H. bacteriophora*. Due to the growth of the symbiotic bacterium, which produces a red pigment (Forst and Clarke, 2002), the cadavers turn red. All cadavers, which had not turned red, were dissected and searched for the presence of EPN. If 1 and/or 2 larvae in each baiting box were infected with nematodes, the soil sample was recorded as positive for the occurrence of EPN.

2.3.11. Molecular identification of EPNs

For identification of EPN isolated of unknown species these strains were subjected to a molecular identification based on a PCR-RFLP of the ITS region (Internal Transcribed Spacer) of the ribosomal DNA.

Total genomic DNA was isolated with a DNeasy® Kit supplied by the Qiagen GmbH (Hilden, Germany) following the instructions of the supplier (Qiagen, 2002). A 200 µl nematode sample (about 2,500-3,500 IJs) was used for DNA extraction in a 2 ml Eppendorf reaction tube. Afterwards extraction, DNA was stored in EtOH (98%) at –27 °C until using.

PCR amplification of the ITS region was carried out in a reaction volume of 100 µl for each strain, containing 75.6 µl of H_2O, 2 µl of dNTPs (2mM), 10 µl of 10 x PCR-Buffer, 1 µl of Primer Forward (200 µM), 1 µl of Primer Reverse (200µM), 0.4 µl of Taq polymerase (5U/µl) and 10 µl of purified DNA. The primers 18S (5`-TTGATTACGTCCCTGCCCTTT-3`) and 26S (5`-TTTCACTCGCCGTTACTAAGG-3`) were used as forward and reverse primers, respectively (Vrain et al., 1992). The amplification was carried out using a DNA thermal cycler (Perkin Elmer Cetus, Emeryville, CA, USA). The samples were placed in the thermal cycler, which was preheated to 95 °C and incubated at 94 °C for 3 min. Amplification was started at an annealing temperatures for 1 min at 55 °C followed by an extension period at 72 °C for 1 min 30 s and a denaturalisation period at 94 °C for 30 s´. After 40 of these cycles, a final step of 1 min at 94 °C, 3 min annealing temperatures at 55 °C and 5 min at 72 °C of extension to ensure that all of the final amplification products were full length (Reid and Hominick, 1998). At the end of the 40 cycles, the samples were stored at 4 °C.

The digestions with different restriction enzymes to produce RFLPs (Restriction Fragment Length Polymorphism) was carried at 37 °C for 3 hours in the thermal cycler using the corresponding restriction buffers with approximately 200 µl of a PCR product. Nine different enzymes were used: Alu I, Hae III, Hind III, Dde I, Hha I, Hinf I, Hpa II, Rsa I and Sau 3Al (Amersham Biosciences, Freiburg, Germany). Each cap containing 1 µl enzyme, 3 µl its buffer and 4 µl aqua dest., except for Hind III, which was used at 0.5 µl Hind III, 3 µl of its buffer and 4.5 µl aqua dest and Hinf I at 1.25 µl enzyme, 3 µl of its buffer and 3.75 µl aqua dest.

The resulting fragments were separated by electrophoresis with a 2 % agarose gel containing ethidium bromide (0.2 %) in 0.5 x TBE buffer (54.0 g Tris base + 27.5 g Boric acid + 20 ml 0.5 M EDTA (pH 8.0) to 1 l aqua dest.). Five µl of a DNA product was mixed with 3 µl loading suspension (30 % glycerol, 50 mM EDTA, 0.001 % bromophenol blue and 0,001 % xylencyanol) and 2 µl aqua dest and transferred into the gel pockets. One pocket was loaded with 5 µl of a marker ranging from 100 to 1,000 base pairs (Smart Ladder SF, Eurogentec, Köln, Germany), which had been treated like the DNA sample. Extracted or amplified DNA was subjected to 180 V for 35 minutes, whereas RFLP products were run at 100 V for 90 min. The fragments were photographed under UV light. RFLP patterns were compared with published information on described species (Reid and Hominick, 1998).

2.4. Field establishment and persistence

To investigate establishment and persistence of applied nematodes in agriculture ecosystems, soil samples were checked for the presence of EPN. Between October 23, 2001 and August 18, 2004 the total number of 10,980 soil samples (1,250 before and 9,730 after EPN application) were collected and subjected to the insect baiting method to detect the presence of EPNs.

2.4.1. Location of experimental fields

To evaluate establishment and persistence, EPN were applied in the field and afterwards soil samples were investigated for the presence of EPN. The experiments on establishment and persistence were carried out at two different locations. One of them is the Lindhof (LiH), which belongs to the Faculty of Agriculture and Nutritional Sciences, University Kiel and is

located 26 km northern of Kiel. The farm is following organic agriculture practice since 1997. The other location is a farm owned by Joachim Postel situated in Rastorfer Passau (RaPa) located 20 km south east of Kiel and is a conventionally managed farm (Tab. 1).

Table 1. Description of experimental locations.

	Lindhof	**Rastorfer Passau**
Cultivation area (ha)	123.5	66
Agricultural management	Organic farming	Conventional farming
Annual precipitation	670 mm	750 mm[1]
Average annual temperature	8.5 °C	8.5 °C
Soil type	Loamy sand	Sandy loam
Geographical location	54° 23' northern latitude	54° 12' northern latitude
	9° 56' southern longitude	10° 19' southern longitude

2.4.2. Influence of application time and crop on establishment and persistence

In order to investigate the influence of application time and crop on nematode establishment, *H. bacteriophora* was applied as described in 2.3.3. on different crops and during different times of the year as indicated in Tab. 2. Applications were made once per month from March until July 2004 at both locations. The height of the crop plants was recorded at the time of nematode application. Each time new plots were sprayed. Prior to spraying, 40 soil samples per plot were taken to check the presence of indigenous EPN. Immediately after application another 40 soil samples per plot were taken and then again in monthly intervals until September 2004. From untreated plots 30 soil samples were taken at the same time when treated plots were investigated. Soil samples were checked for the presence of indigenous EPN (controls and samples taken before spraying) and for the applied strain of *H. bacteriophora* according to the method described in 2.3.4. Climatic conditions were recorded at the experimental fields during the period of investigations and the data on air and soil temperature, relative humidity, precipitation and global radiation are presented in Annex 2 (Lindhof) and Annex 3 (Rastorfer Passau).

[1] www.klimadiagramme.de/Deutschland/Plots/kiel_3.gif

2.4.3. Long-term persistence of *S. feltiae* and *H. bacteriophora*

Nematode persistence was investigated by checking soil samples from treated and untreated plots up to a period of maximum 3 years for the presence of EPN. Application was done as described in 2.3.3., except that applications done on plots of 18 m x 500 m with commercial sprayers provided by the farmers at the Lindhof in October 2001 and at Rastorfer Passau in June 2003. Applications were made according to details presented in Tab. 3. Prior to spraying, 40-50 soil samples per plot were taken to check for the presence of indigenous EPN. Immediately after application again another 40-50 soil samples per plot were taken. Soil samples were checked for the presence of indigenous EPN (controls and samples taken before spraying) and for the applied EPN strains according to the method described in 2.3.4. Whenever nematodes were detected from soil samples taken before application or from untreated control plots, these were subjected to a molecular analysis as described in 2.4.5. in order to identify the species of the indigenous isolates.

2.5. Laboratory studies on persistence

Investigations on the persistence of nematodes in the field are influenced by the availability of potential host insects. Thus persistence studies cannot distinguish between the recovery of a released population and the recovery of offspring of the released population. To exclude this factor, experiments on nematode persistence were conducted in the laboratory to assure that no host insects can influence the results. In a first experiment the persistence of *S. feltiae* and *H. bacteriophora* was investigated at 8°C. The nematodes were applied at a concentration of 50 IJs cm^{-2}. The experiment was carried out in pots of 12 x 12 x 12 cm filled with soil from the Lindhof. Prior to the experiments the soil had been baited to assure that no indigenous nematode population was present. Sterile water was added to the soil to achieve water content of 10% (w/v). The soil moisture was checked with electronic moister analyser (Sartorius MA 40, Göttingen, Germany). An amount of 7,200 IJs were applied per pot and incubated for 12 weeks. For each nematode species, 20 pots were used. Every 2 weeks one soil sample of approximately 3 cm^2 and 40 g was taken from each pot with a soil borer (2 cm diameter and 12 cm height).

Table 2. Establishment and persistence of 2004 applied *H. bacteriophora* with dates of applications and crop on which the nematode have been sprayed.

Appl. Time	Plants				Farms
24.03.2004	Oil seed rape (*Brassica napus*)	Pasture	Wheat (*Triticum durum*)	-	Rastorfer Passau
05.04.2004	Clover (*Trifolium pratense*)	Barley (*Hordeum vulgare*)	-	-	Lindhof
27.04.2004	Oil seed rape (*Brassica napus*)	Pasture	Wheat (*Triticum durum*)	-	Rastorfer Passau
11.05.2004	Clover (*Trifolium pratense*)	Pea (*Pisum sativum*)	Barley (*Hordeum vulgare)*	-	Lindhof
01.06.2004	-	Pasture	Wheat (*Triticum durum*)	Corn (*Zea mays*)	Rastorfer Passau
07.06.2004	Clover (*Trifolium pratense*)	Pea (*Pisum sativum*)	Potato (*Solanum tuberosum*)	Barley (*Hordeum vulgare)*	Lindhof
02.07.2004	-	Pasture	Wheat (*Triticum durum*)	Corn (*Zea mays*)	Rastorfer Passau
15.07.2004	Clover (*Trifolium pratense*)	Pea (*Pisum sativum*)	Potato (*Solanum tuberosum*)	Barley (*Hordeum vulgare)*	Lindhof

Table 3. Dates of EPN applications, crops on which EPNs were sprayed and following crops in the rotation.

Appl. Time	EPNs	Crop rotations 2000	2001	2002	2003	2004	Farm
23.10.2001	*S. feltiae*	Oil seed rape (*Brassica napus*)	Clover (*Trifolium pratense*)	Oat (*Avena sativa*)	Potato (*Solanum tuberosum*)	Pea (*Pisum sativum*)	Lindhof
23.10.2001	*H. bacteriophora*	Pea (*Pisum sativum*)	Oil seed rape (*Brassica napus*)	Clover (*Trifolium pratense*)	Oat (*Avena sativa*)	Wheat (*Triticum durum*)	Lindhof
11.06.2002	*H. bacteriophora*	-	Oat (*Avena sativa*)	Bean (*Vicia faba*)	Winter Wheat (*Triticum durum*)	Clover (*Trifolium pratense*)	Lindhof
19.06.2003	*H. bacteriophora*	-	-	Clover (*Trifolium pratense*)	Wheat (*Triticum durum*)	Barley (*Hordeum vulgare*)	Rastorfer Passau
19.06.2003	*H. bacteriophora*	-	-	Pasture	Pasture	Pasture	Rastorfer Passau
19.06.2003	*H. bacteriophora*	-	-	Clover (*Trifolium pratense*)	Oil seed rape (*Brassica napus*)	Wheat (*Triticum durum*)	Rastorfer Passau
08.07.2003	*H. bacteriophora*	-	-	Potato (*Solanum tuberosum*)	Lupin (*Lupinus angustifolius*)	Pea (*Pisum sativum*)	Lindhof
08.07.2003	*H. bacteriophora*	-	-	Oat (*Avena sativa*)	Potato (*Solanum tuberosum*)	Pea (*Pisum sativum*)	Lindhof
08.07.2003	*H. bacteriophora*	-	-	Wheat (*Triticum durum*)	Pea (*Pisum sativum*)	Potato (*Solanum tuberosum*)	Lindhof
11.11.2003	*S. feltiae*	-	-	-	Oil seed rape (*Brassica napus*)	Oil seed rape (*Brassica napus*)	Rastorfer Passau

Two *G. mellonella* were added to the each sample to bait the nematodes. Baiting was repeated three times with 3-day intervals. The infected larvae were dissected and the number of IJs per insect was counted. Therefore, dead *G. mellonella* were washed in Ringer´s solution in order to remove EPNs on the surface of the insect and the cuticle of the larva was removed with the help of scissors and pins. The haemolymph was homogenized by consecutive passages of the haemolymph together with 2 ml Ringer`s solution through a Pasteur pipette. The suspension was then poured into a 10 ml glass tube and centrifuged at 2000 rpm for 2 min. After centrifugation, the supernatant was carefully removed and the sediment containing the nematodes was diluted by adding Ringer´s solution at a ratio of 1:5. All IJs were counted in counting cell-wells under the microscope and the mean half-life of EPNs was calculated.

In a second experiment sterilized and non-sterile soil from both locations were used at 15 and 25°C to assess the persistence of *H. bacteriophora* over a period of 9 weeks. The monitoring was carried out parallel to the field trials in 2004. The soil samples were collected from an oil-seed rape field in Rastorfer Passau (RaPa) and a potato field from the Lindhof (LiH) in April 2004. The collected samples were divided and one part was sterilised at 80 °C for 12 h. After sterilisation, samples were supplemented with tap water and in all samples the moistures were measured using the electronic moister analyser. Each of the sterile (s) and un-sterile (us) samples (LiH-s, LiH-us, RaPa-s and RaPa-us) was separated and subjected to the experimental temperatures of 15 and 25 °C. Approximately 60 g soil sample was transferred to plastic boxes of 5 x 5 x 5 cm (100 ml) and incubated for one day. At the following day, *H. bacteriophora* was applied into each box. Six boxes of each variant were examined immediately and then 1, 3, 5, 7 and 9 weeks after the application. Finally, recovered IJs were dissected and counted with the method described previously.

2.6. Characterisation of re-isolated *Heterorhabditis bacteriophora*

In order to investigate whether the fitness of the released nematodes will change within one year after application in the field, IJs infectivity (2.3.5.), reproduction capacity (2.3.6.) and the potential to persistence (2.5.) were compared between a *H. bacteriophora* population re-isolated from plots where the strain had been released in June 2002 in beans at the Lindhof and a population taken from *in vitro* mass production provided by e-nema GmbH. Prior to comparison between the samples of the different sources the newly fermented nematodes were reproduced once in *G. mellonella* larvae in order to achieve equal conditions. Persistence

was evaluated according to the first method described in 2.5. at 16 °C over a period of 12 weeks.

2.7. Persistence and efficacy of *H. bacteriophora* against *Otiorhynchus sulcatus*

Strawberries under plastic mulch nematodes cannot be applied when the pest *O. sulcatus* produces major damage. Should nematodes be able to persist over a period of two months and then still cause sufficient control of the pest, an application during planting would be possible. To evaluate the control potential of *H. bacteriophora* applied by dipping roots of transplants, the following experiment was conducted. A nematode suspension with 1,500 IJs/ml of *H. bacteriophora* was supplemented with wallpaper glue Methylan (Henkel AG, Düsseldorf) at 0.5 %. The glue contains carboxy-methyl-cellulose (CMC), which is added to prevent nematodes from settling and to stick them to the roots of Frigo plants (Peters et al., 2002). The root system of one Frigo plant adsorbs approximately 4-5 ml of this suspension that each plant received approximately 7,000 IJs. In total, 120 of 150 strawberry plants (weight 14-15 g) were dipped into the nematode suspension (Fig. 4). Before and after dipping, the plants were weighed in order to detect how much ml solution remained on the roots. The weight of each plant was recorded and every pot was numbered. Thirty plants were dipped into tap water only to be used as untreated controls. The plants were then transferred to 1 l pots of 12 x 12 x 14 cm (L x W x H) containing a soil mixture of 337.5 g (45 %) compost, 337.5 g (45 %) sandy-loam soil, 67.5 g (9 %) silver sand and 7.5 g (1 %) fertilise for strawberry. Prior to mixture, the components had been sterilised at 80 °C for 12 h. After sterilisation, all components were gently mixed and adjusted to 10 % moisture with sterile water. All pots were kept under controlled condition at variable temperature from 18 °C at night to 26 °C during the day with a photoperiod of light: dark = 16:8 h.

Immediately after dipping and in 2 weeks intervals, 5 nematode-treated pots were supplemented with 20 larvae of *T. molitor* and incubated at 25 °C for 1 week to investigate persistence. The number of nematode-infected larvae was recorded. Three weeks after planting, 95 dipped plants were treated with 30 eggs of *O. sulcatus* per pot. The eggs were added by using a soft brush (No: 2) and deposited at 3-4 mm depth of the potting substrate.

Figure 4. Root system of a Frigo strawberry plant dipped into a nematode suspension planting into a pot.

The addition of egg into the pots was replicated every month. However, in the second and third egg inoculation only 20 eggs were transferred into the pots. One month after the eggs had been transferred, 10 pots from the nematode treatment and the same amount of pots, which were free of nematodes, were carefully checked for the presence of *O. sulcatus* larvae. Larval length was measured by analysing images with the software program Analysis® (Soft Imaging System) and data were compared with published results (van Tol et al., 2001) to determine the larval instar. This procedure was continued every month until 3 months after the addition of eggs. Persistence evaluated by the addition of 20 *T. molitor* was also recorded in 5 pots every two weeks, which had received nematodes and eggs of *O. sulcatus* (Peters et al., 2002).

2.8. Persistence and efficacy of *Steinernema feltiae* against *Delia radicum*

Larvae of the cabbage root fly are most susceptible to *S. feltiae* during the third instar (Sulistyanto et al., 1994). This stage was detected in a field of oil-seed rape at the farm in RaPa in autumn 2003. Field persistence of *S. feltiae* was assessed at that field. To get data on nematode efficacy and persistence under more controlled conditions, the following experiments were conducted: Plants at EC 22 were collected from the field on November 13, 2003 and transplanted into pots of 12x12x14 cm (L x W x H) and supplemented with 3 field collected third instar *D. radicum* per plant. All pots were incubated at 8 °C for one day. After the incubation, half of the pots were treated with *S. feltiae* at a dose of 50 IJs cm^{-2}. The other 25 plants were used as controls. Once every 2 weeks starting 2 weeks after nematode

application, the soil of each pot was checked for the presence of larvae and pupae. This procedure was continued until no larvae were detected or had pupated. The experiment was carried out at 8 °C.

In order to measure persistence of *S. feltiae,* samples were taken and subjected to baiting with *G. mellonella* as described under 2.5. This procedure was continued over a period of 12 weeks. The laboratory experiments were synchronised with the field assessments for persistence.

In an additional experiment it was investigated whether *S. feltiae* could identify *D. radicum* larvae over a longer distance and migrate towards its host and whether nematodes are also attracted to oil-seed rape roots. A Y-tube Olfactometer as presented in Figure 3 was used. The arena consisted of a dark grey hard PVC Y-connection used in pluming for sewage water disposal. All three arms were filled with fine sterilised silver sand (10% w/v moisture).

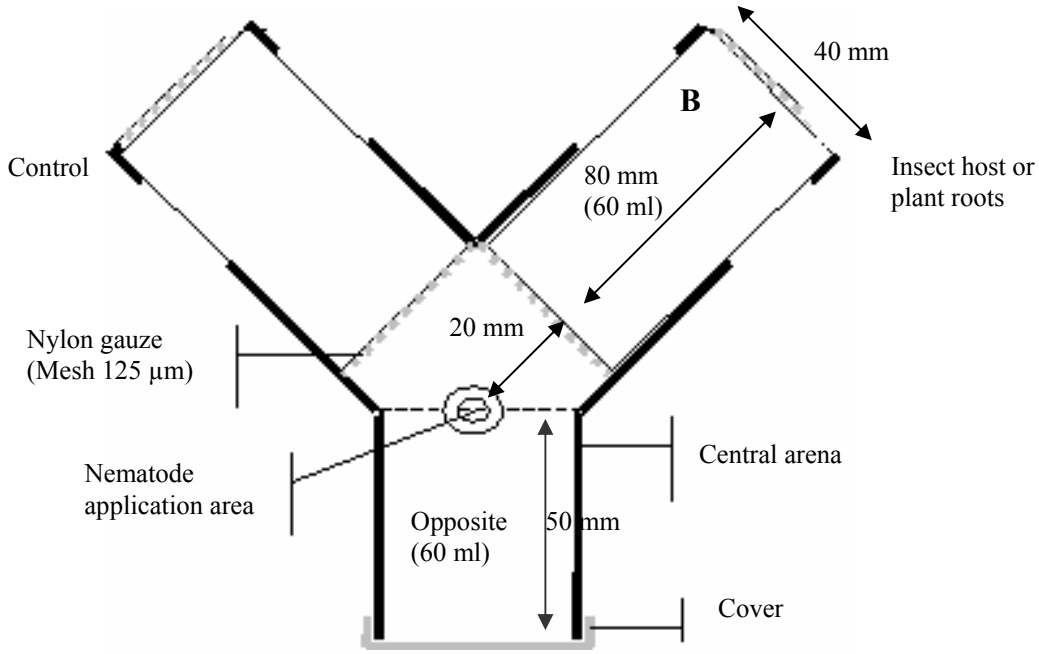

Figure 5. Schematic drawing of the Y-Olfactometer choice arena for recording preferential responses of EPNs to insect and root stimuli in sand (modified from Boff et al., 2001).

The behavioural response of IJs of *S. feltiae* to the presence of 5 last instar *D. radicum* or to roots of one oil-seed rape plant was investigated. In a choice experiment 5 larvae of *D. radicum* were offered in one arm and oil-seed rape roots in the other. All three experiments were done in parallel with three olfactometers. The larvae were kept in a sieve of 1 mm mesh size to prevent them from migrating through the tube. Before adding nematodes, the assay

units were incubated in a horizontal position in the dark room at 8 and 15 °C for 24 h to allow the formation of a chemical gradient. Then one ml of Ringer´s solution containing 1,000 IJs (±15 %) was added to the centre hole of 50 mm of the nematode application area (Fig. 5) and the olfactometers were incubated for 10 days at the above temperatures. After this period, the olfactormeter was taken apart and the nematodes were recovered from the sand by using a modified Cobb`s (1918) decanting and sieving method (Klein-Beekman et al., 1994). The amount of sand of each compartment was put into a 1 l litre glass cylinder and 600 ml tap water was poured over it. After 10 seconds, the supernatant was poured into a 2 litres beaker. The remaining sand was mixed with 600 ml of top water for another two times, but now the supernatant was collected and added to the beaker suspension after 15 seconds. The total beaker suspension with the nematodes was poured through a sieve (mesh size 0.01 mm). The nematodes were remaining on the sieve and were collected in a small beaker. The nematodes recovered from the sand of each compartment of the Y-Olfactometer were counted in a counting plate under a microscope. The insect larvae were dissected and the number of IJs was determined.

2.9. Statistical analyses

In efficacy experiment with EPNs, first, total insect mortality data were corrected for control mortality using Abbott`s formal (Abbott, 1925). Mortality comparisons between in treatment and control were evaluated by the Mann Whitney test at $p<0.05$.

Distribution of *S. feltiae* in Y-Olfactometer tubes was analysed by analysis of variance ANOVA (breakdown one way Anova) and followed by a Least Significant Difference (LSD) test as post-hoc comparisons of the mortality means. The minimum level of significance was taken as $p<0.05$.

Prior to each application in the field in 2004, $LD_{50,}$ LD_{90} and LT_{50} of *H. bacteriophora* were estimated by Probit analysis (2.3.4.) according to Finney (1971).

The correlation was tested between the height of the crops and the number of IJs cm-2 recovered in Petri dishes (2.4.2). The establishment (percent of positive samples) and the amount of precipitation during the application week were also estimated. Correlation test were calculated with $p < 0.05$.

In establishment experiments in the fields, differences of percent of detected positive soil samples immediately after applications for each crop were compared using Chi²-Test. The applied test examined whether the number of positive and/or negative soil samples is dependent on the treatment (application date and/or crop as variables). The same test was used in order to examine whether the percentage of positive soil samples between different application dates in one crops were significantly different.

Data obtained from persistence experiment in the laboratory on number of IJs obtained from sterile and un-sterile soils were subjected to the Harder test at $p<0.05$.

The statistic analysis was performed by using of the program Statgraphics, Statistica and Xlstat Pro 7.0.

3. Results

3.1. Indigenous EPN populations in the experimental fields

In order to detect indigenous EPN populations in the experimental fields at the Lindhof and in Rastorfer Passau, soil samples were collected before nematode application (2.3.9.) and subjected to the baiting methods (2.3.10.). Isolates were identified by molecular analysis of the ITS region of the ribosomal DNA (2.3.11.). As an example the RFLP pattern of the ITS region of the ribosomal DNA of the isolate from oil seed rape sampled on October 23, 2001 is presented in Fig. 6. The only species detected in soil samples was *S. feltiae.* Before EPN application, a total number of 1,250 soil samples was taken and analysed between 2001 and 2004 from 19 fields at both locations. *S. feltiae* was detected in only 22 out of 1,250 soil samples (1.76 %). Results are summarized in Tab. 4.

Table 4. Records of EPN positive soil samples taken before the release of nematodes between 2001 and 2004. From each field 50 soil samples had been taken.

Time of soil sampling	Crop	Prior crop	Farm	Species	No. of positive soil samples, (%)
23.10.01	Clover (*Trifolium pratense*)	Oil-seed rape (*Brassica napus*)	Lindhof	*S. feltiae*	6 (12%)
23.10.01	Oil-seed rape (*Brassica napus*)	Pea (*Pisum sativum*)	Lindhof	*S. feltiae*	3 (6%)
11.06.02	Bean (*Vicia faba*)	Oat (*Avena sativa*)	Lindhof	*S. feltiae*	8 (16%)
24.03.04	Pasture	Pasture	Rastorfer Passau	*S. feltiae*	3 (6%)
27.04.04	Pasture	Pasture	Rastorfer Passau	*S. feltiae*	1 (2%)
11.05.04	Clover (*Trifolium pratense*)	Winter Wheat (*Triticum durum*)	Lindhof	*S. feltiae*	1 (2%)

3.2. Competition between indigenous and applied EPN species

Whether an indigenous species is replaced by an introduced species could easily be investigated by application of *H. bacteriophora*, as this species was never detected in any of the soil samples and therefore could be distinguished from indigenous *S. feltiae* populations. Of the six fields in which indigenous *S. feltiae* were detected, five were afterwards treated with *H. bacteriophora*. There was no strong evidence that the indigenous populations were replaced by application of *H. bacteriophora.*

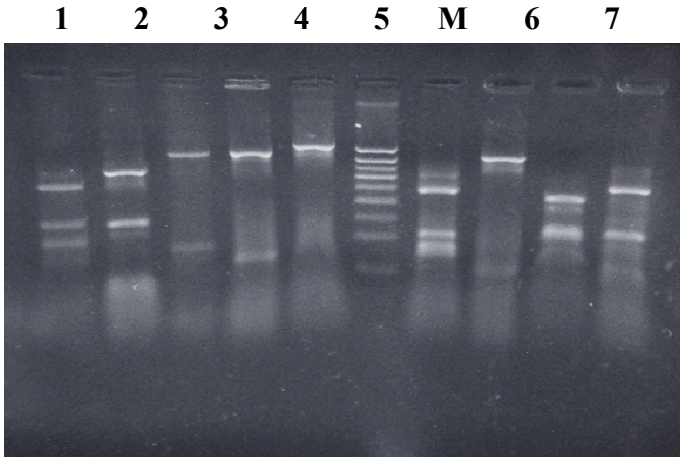

Figure 6. PCR amplified products from the ITS region of *S. feltiae* digested with 9 restriction enzymes. Lanes **1-9** indicate the following enzymes: **1**. Alu I; **2**. Dde I; **3**. Hae III; **4**. Hha I; **5**. Hind III; **M**. Molecular weight markers (band sizes 1000, 800, 700, 600, 500, 400, 300, 200, 100 base pairs) **6**. Hinf I; **7**. Hpa II; **8**. Rsa I (Afa I); **9**. Sau 3 AI.

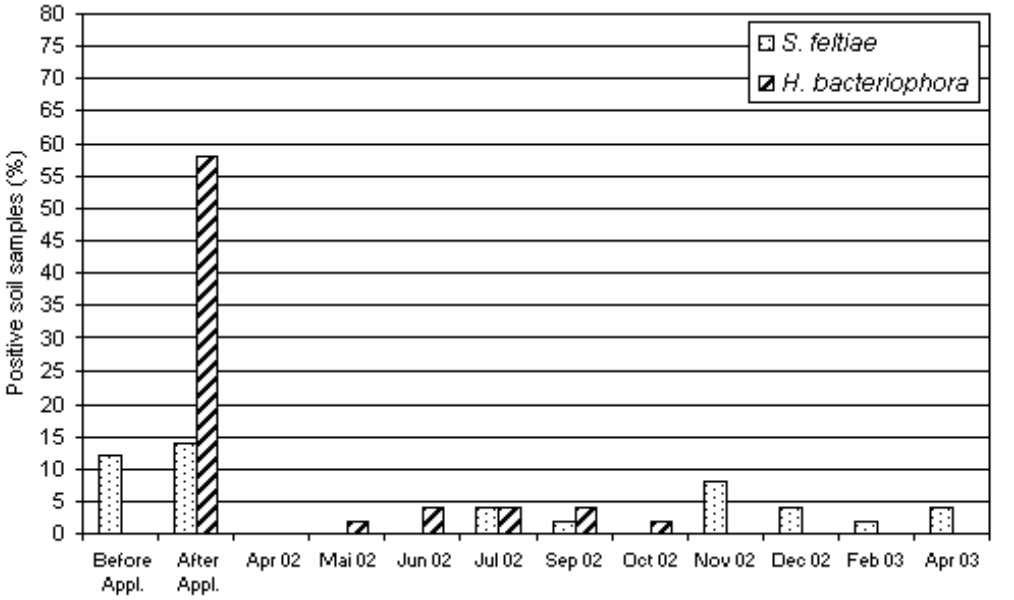

Figure 7. Percentage of soil samples positive for an indigenous population of *S. feltiae* and the released species *H. bacteriophora* applied on 23.10.2001 in clover (*Trifolium pratense*) on the Lindhof. No EPNs were detected after April 2003.

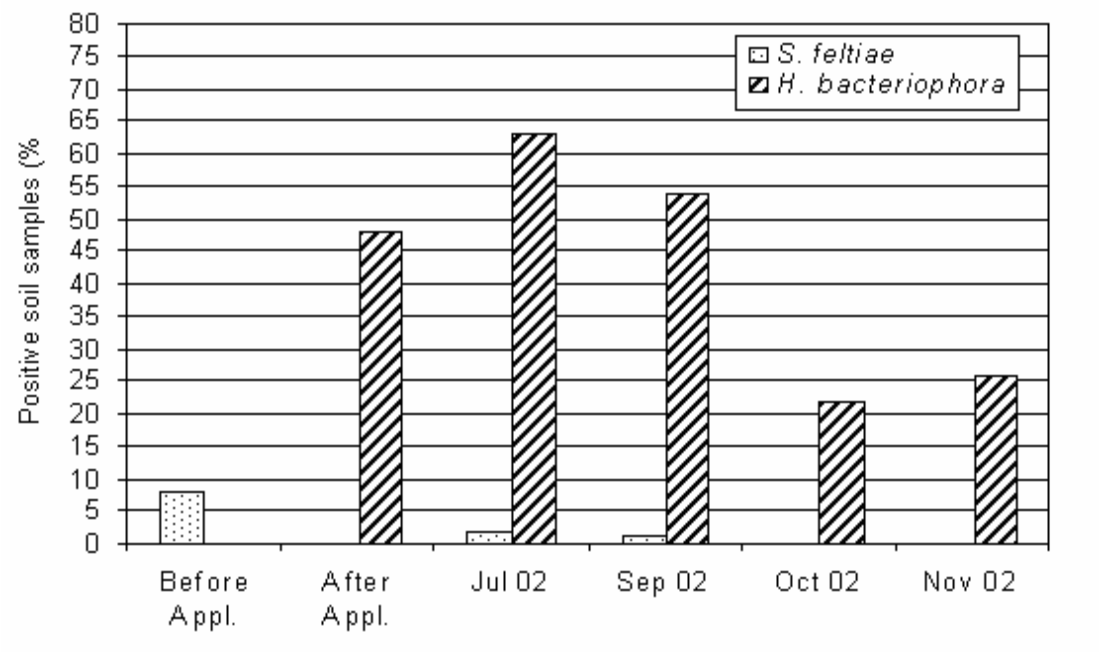

Figure 8. Percentage of soil samples positive for an indigenous population of *S. feltiae* and the released species *H. bacteriophora* applied on 11.06.2002 in beans (*Vicia faba*) on the Lindhof. No *S. feltiae* was detected after November 2002.

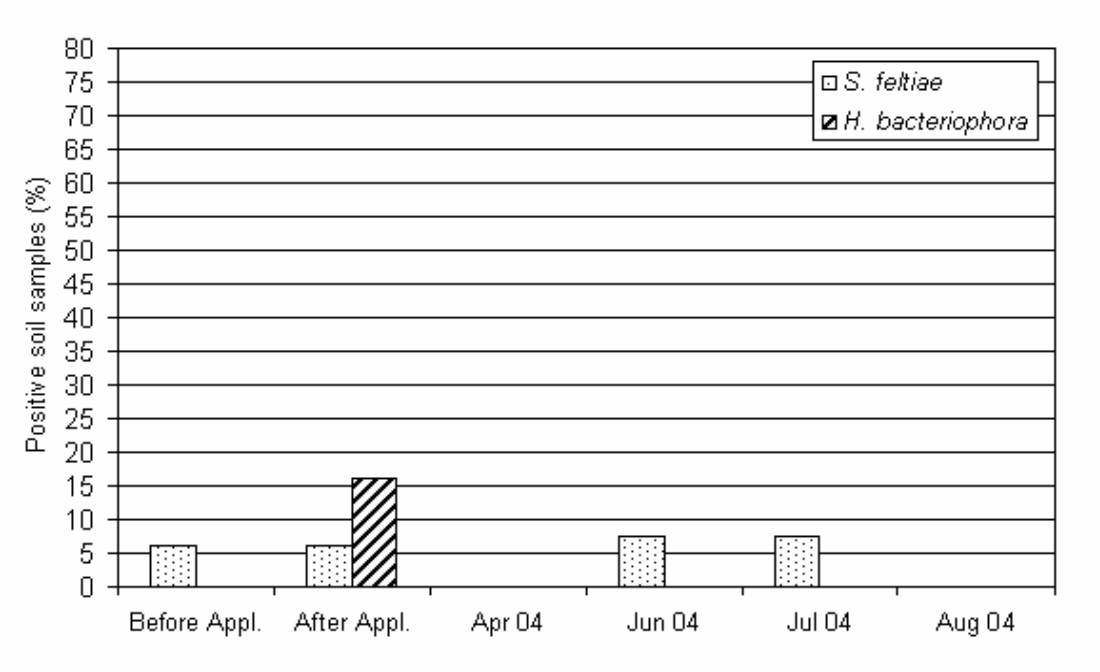

Figure 9. Percentage of soil samples positive for an indigenous population of *S. feltiae* and the released species *H. bacteriophora* applied on 24.03.2004 in pasture in Rastorfer Passau.

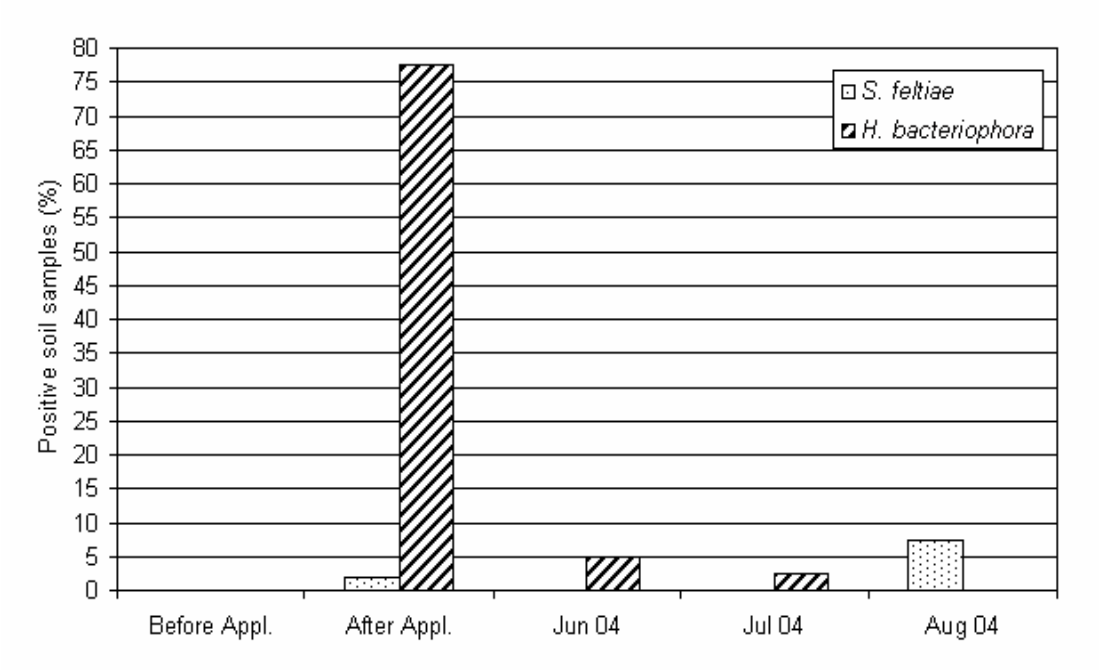

Figure 10. Percentage of soil samples positive for an indigenous population of *S. feltiae* and the released species *H. bacteriophora* applied on 27.04.2004 in pasture in Rastorfer Passau.

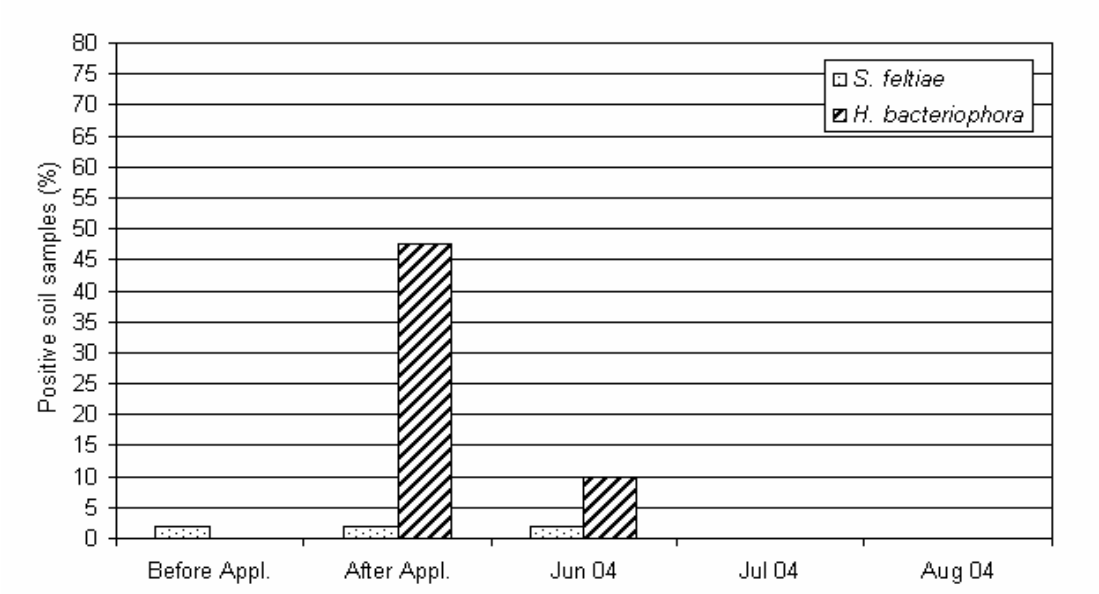

Figure 11. Percentage of soil samples positive for an indigenous population of *S. feltiae* and the released species *H. bacteriophora* applied on 11.05.2004 in clover (*Trifolium pratense*) on the Lindhof.

Results presented in Figures 7, 9 and 11 indicate that *S. feltiae* was not affected by the presence of the released *H. bacteriophora*. In the applications, which were done on March 24, 2004 and April 27, 2004 in pasture fields the incidence of positive samples of the inoculated *H. bacteriophora* was rapidly reduced and fell under the level of positive samples with S.

feltiae. In these fields *H. bacteriophora* disappeared 1 and 4 months after application (Fig. 9 and 10). On the field in which *H. bacteriophora* was applied in June 2002, the population of *S. feltiae* could not be isolated 4 months after the release of *H. bacteriophora* (Fig. 8). In contrast to the other fields, this field suffered from an infestation with larvae of pea weevil, *Sitona lineatus* L. (Coleoptera: Curculionidae), a suitable host of *H. bacteriophora* (see also 3.5.). The occurrence of suitable hosts might be one reason why the population of *H. bacteriophora* was dominant over *S. feltiae.* However, *S. feltiae* has a wide host range including other curculionids and it is one of the EPN species, which is rather non-specific (Peters, 1996). It can therefore be speculated that *Sitona* sp. might also serve as host for *S. feltiae* and results by Wiech and Jaworska (1990) have shown that *Sitona* spp. can serve as hosts for both species. Then *H. bacteriophora* can only dominate if it has a larger capacity to exploit this host. However, more detailed investigations are needed to draw final conclusions on competition between EPN species.

In the other fields, no insect pests were detected during the soil sampling. Therefore, the population of the indigenous *S. feltiae* might have persisted in the presence of *H. bacteriophora*. In one field *S. feltiae* was replaced by *H. bacteriophora* but was again isolated in the month when no *H. bacteriophora* could be detected (Fig. 10). This observation together with the results of the other fields where *S. feltiae* was continuously found indicates that it is possible for the indigenous *S. feltiae* to co-exist with a released population of *H. bacteriophora.*

3.3. Persistence of inoculated EPNs in the field

The nematode species *S. feltiae* and *H. bacteriophora* were applied in described fields between October 2001 and August 2004. Each field was only once treated with these nematodes and the number of positive soil samples was monitored during the following months after application. In all cases the released nematodes were at least once successfully recovered from the fields by using of insect bait technique with *G. mellonella* (2.3.10.). A total number of 6,680 of soil samples (470 before + 6,210 after application) were collected and baited almost every month, except when adverse weather conditions (generally in December, January and February) prohibited the sampling. The longest persistence of 23 months was recorded for a release of *H. bacteriophora* in beans (Fig. 12). The results indicate

that *H. bacteriophora* has the potential to persist for many months in agriculture ecosystems, however, in none of the release locations it could be consistently established.

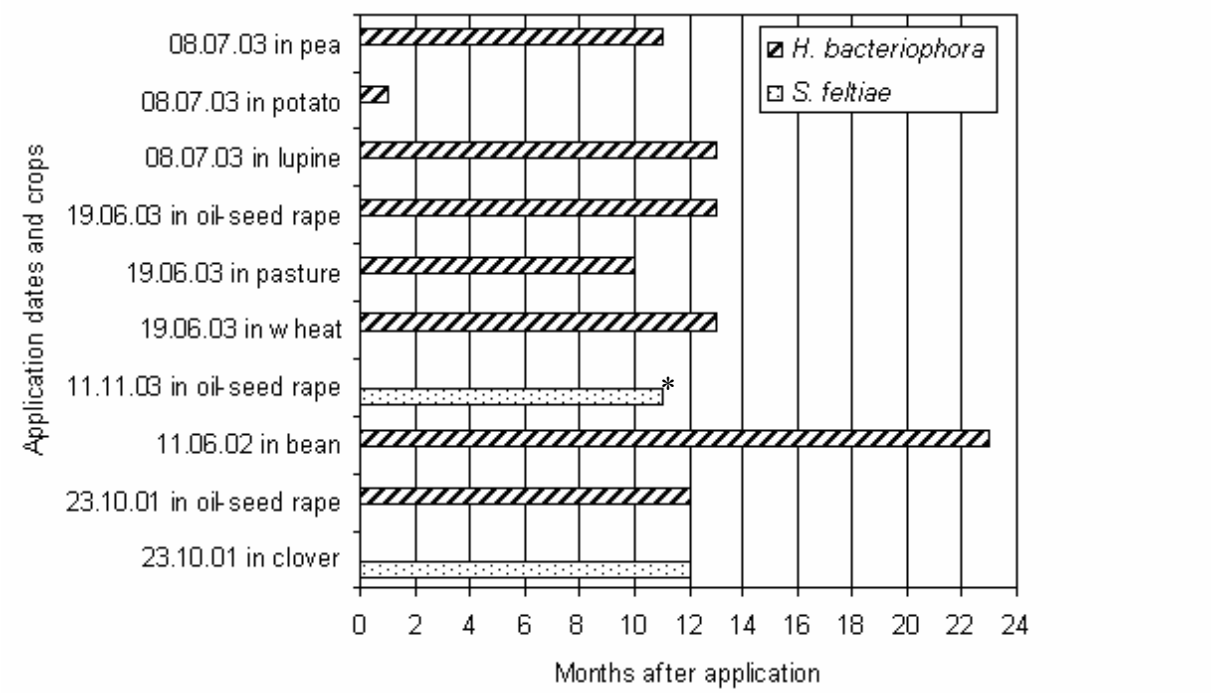

Figure 12. Persistence measured as soil samples positive for the released species (even one positive soil sample) *S. feltiae* and *H. bacteriophora* applied at different times and in different crops. * When the investigations were terminated, *S. feltiae* was still present in the samples (n = 650 for applications on 08.07. and 19.06.03; n = 160 for 11.11.03; n = 950 for 11.06.02 and n = 600 for 23.10.01 applications).

Figure 12 demonstrated that although *H. bacteriophora* was applied in pea, potato and lupine at the same time, the persistence is highly variable in these crops. The crops and/or the phytophagous host insects probably play a key role in the persistence of the nematode variability. But quality of the nematode material and the plant's architecture and height might also have influenced establishment.

To exclude that the quality of the released nematode population might have influenced establishment and subsequent persistence, survival and fitness of the released population was recorded. Before application, 100 µl nematode suspensions were taken from the spray tank and nematodes were counted under the microscope to assess the proportion of living individuals (2.3.2.). This procedure was also applied to the nematodes, which were recovered from Petri dish used to sample nematodes, which had been passing the sprayer. The sampled nematodes were then subjected to a bioassay with *G. mellonella* larvae to test their fitness (2.3.3.)

Table 5. Survival and pathogenicity (Patho.) of the released nematodes recorded before and after application (Appl.).

Appl. Dates	Nematode species	Survival (%)			Pathogenicity (%)		
		Before appl.	After appl.	Survival rate (Before appl./ After appl.)	Before appl.	After appl.	Patho. rate (Before appl./ After appl.)
23.10.2001	*S. feltiae*	92.6±2.8 [a] A	90±3.7 [a] A	0.97	84.8±3.7 [a] A	84.6±4.4 [a] A	0.99
11.06.2002	*H. bacteriophora*	89.8±5.8 [b] A	85±6.4 [b] A	0.94	83±4.7 [b] A	79±3.6 [b] A	0.95
19.06.2003	*H. bacteriophora*	89.4±3.4 [c] A	83.6±4.0 [c] B	0.94	89.2±9.4 [c] A	86.6±7.6 [c] A	0.97
08.07.2003	*H. bacteriophora*	95.6±5.6 [d] A	94.3±6.8 [d] A	0.98	88±10 [d] A	85±6.5 [d] A	0.96
11.11.2003	*S. feltiae*	94.6±6.3 [e] A	92.3±3.3 [e] A	0.97	89±11 [e] A	82±9.2 [e] A	0.92

Note: Data present mean ± SE. Refer to small letters for F-quartiles, degree of freedom and probability. Means within the row (before and after application in survival or pathogenicity) followed by the same capital letter are not significantly different ($p<0.05$) according to the Least Significant Differences (LSD) test. Pathogenicity (%) was calculated considering mortality in the controls using Abbott`s formula (Abbott, 1925).

Survival*:* [a] ANOVA: F=1.5504; df=1, 8; p=0.2483, [b] ANOVA F=1.5426; df=1, 8; p=0.2494, [c] ANOVA: F=5.9857; df=1,8; p=0.0401, [d] ANOVA: F=5.9857; df=1, 8; p=0.0401, [e] ANOVA: F=0.5818; df=1, 8; p=0.4674.

Pathogenicity: [a] ANOVA: F=0.0060; df=1, 8; p=0.9398, [b] ANOVA: F=2.2535; df=1, 8; p=0.1717, [c] ANOVA: F=0.2331; df=1, 8; p=0.6421, [d] ANOVA: F=0.3092; df=1, 8; p=0.5933, [e] ANOVA: F=1.1666; df=1, 8; p=0.3115

According to the results (Tab. 5) there were no significant differences ($p<0.05$) in survival and pathogenicity of nematode material between before and after application, except in survival of the application that was done on June 19, 2003 (F=5.9857; p=0.0401; survival rate=94%). The minimum survival and pathogenicity rates were 94% and 92%, the maximum rates were 97% and 99%, respectively (Tab. 5). Thus the application had a neglectable impact on nematode survival and pathogenicity and should therefore not have influenced the success of the establishment or the subsequent long-term persistence.

Table 6. Data on drop size, number of drops per cm² and number of mean number of IJs per drop obtained after spraying *H. bacteriophora* with the experimental plot sprayer (mean ±SE, n=1920).

No. of drops/cm² (min.-max.)	Diameter of the drops (µm) (min.-max.)	No. of IJs/drop (min.-max.)
6.65±8.89	1212.81±684.8	7.96±1.02
(4.36-9.14)	(240.39-5012.47)	(6.91-8.94)

Data presented in Table 6 show how the standard application dosage of approximately 50 IJs cm^{-2} is distributed over the spraying drops. If the numbers of IJs per drop x numbers of drops per cm² = application dosage then the dosage was 7.96 x 6.65 = 52.934 IJs per cm². Data are means of three independent applications. According to the results, the volume of each drop was approximately 0.934 mm³ so each IJ was applied with approximately 0.12 mm³ water (Tab. 6).

As nematodes were applied to different crop plants, which differed in their developmental stage as well, the plant architecture might have influenced the amount of nematodes, which reached to soil surface. Therefore, the number of the applied nematodes per cm² was assessed by placing Petri dishes below the plant canopy on the soil surface (2.3.7.). A negative correlation was detected between the height and the number of the IJs per cm² (r = - 0.71) recovered in the Petri dishes below the plants. In controls (Petri dishes on bare soil).

Table 7. Data on crops and height of crops, soil and air temperature during application and recovery of EPN under plant canopy and bare soil.

Appl. Dates and times (hour)	Nematode species	Plants	Plant heights (cm)	Soil/air temp.(°C)	IJs/cm²	IJs/cm² in control (without plant)
23.10.01-05:30	*S. feltiae*	Clover (*Trifolium pratense*)	19-25	6.5 / 8	29.8±12.0	48.3±16.0
23.10.01-05:30	*H. bacteriophora*	Oil-seed rape (*Brassica napus*)	9-14	6.5 / 8	38.5±7.5	49.5±12.0
11.06.02-14:00	*H. bacteriophora*	Bean (*Vicia faba*)	58-70	15.5 / 22	45.7±6.8	52.1±18.0
19.06.03-16:30	*H. bacteriophora*	Wheat (*Triticum durum*)	78-90	15.5 / 20	26.2±9.0	53.8±13.5
19.06.03-16:44	*H. bacteriophora*	Pasture	5-7	15.5 / 20	41.8±11.2	54±11.9
19.06.03-17:12	*H. bacteriophora*	Oil-seed rape (*Brassica napus*)	97-110	15.5 / 20	2.3±1.9	49.7±13.9
08.07.03-15:00	*H. bacteriophora*	Lupine (*Lupinus angustifolius*)	90-110	17.5 / 25	3.0±1.8	48.5±9.7
08.07.03-15:17	*H. bacteriophora*	Potato (*Solanum tuberosum*)	65-70	17.5 / 25	42.6±5.3	54.8±18.9
08.07.03-15:42	*H. bacteriophora*	Pea (*Pisum sativum*)	77-91	17.5 / 25	23.6±6.7	53.3±8.0
11.11.03-15:00	*S. feltiae*	Oil-seed rape (*Brassica napus*)	14-17	5 / 7	41.1±13.6	52.9±15.7

The estimated number of approximately 50 IJs cm^{-2} was almost always reached with a minimum number of 48.3± 16.0 and a maximum of 54.8± 18.9 (Tab. 6). Consequently, nematodes must have been lost in the plant canopy. In a latter investigation (3.5.) the correlation between the loss in the plant canopy and the establishment was calculated.

Once the nematodes were applied into the target fields, their population was monitored in the following months after the application (2.3.10.) by exposing soil samples to *G. mellonella* larvae. In the following the results of the long term monitoring of the released populations will be presented.

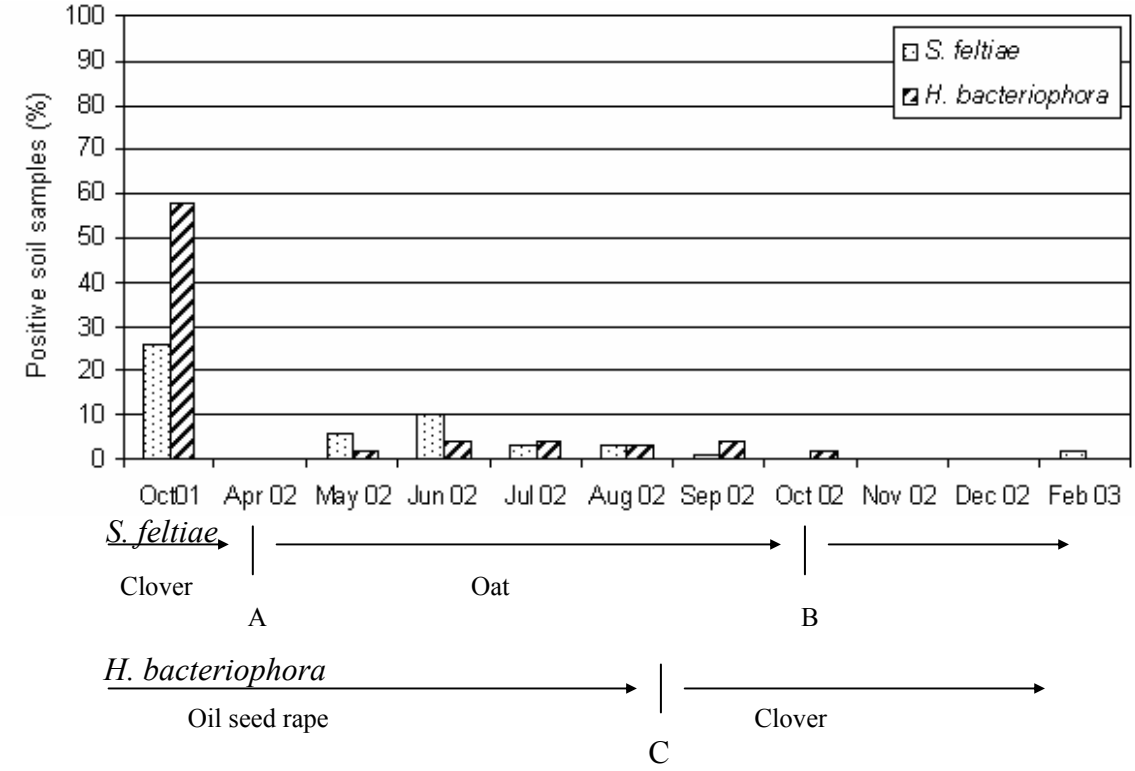

Figure 13. Percentage of soil samples (50 per sampling date and field) positive for *S. feltiae* and *H. bacteriophora* released on 23.10.01 in clover and oil seed rape fields, respectively at the Lindhof. Samples taken on 04.-07., 09. and 10.2003 and 03.-08.2004 were negative. The horizontal arrows indicate crops in the field. No sampling was done in these fields between November 2001 and March 2002. The capital letters indicate tillage. A: Clover was treated with a disc harrow and then ploughed; B: Field was ploughed; C: No tillage was done, red clover was an under-sowing of oil seed rape.

During the months after application, neither *S. feltiae* nor *H. bacteriophora* surpassed a percentage of positive samples of 10%. However, it is surprising that the populations were able to establish as soil temperature, was 6.5 °C at the date of application (Tab. 6). After their applications, 29.8±12 and 38.5±7.5 IJs per cm^2 of *S. feltiae* and *H. bacteriophora*, respectively, were found in the Petri dishes (Tab. 6) into soil. According to the results,

immediately after application, 58% of soil samples were positive for *H. bacteriophora*, considerably higher than for *S. feltiae* (26%). After February 2003 until August 2004 no nematode were found in these fields.

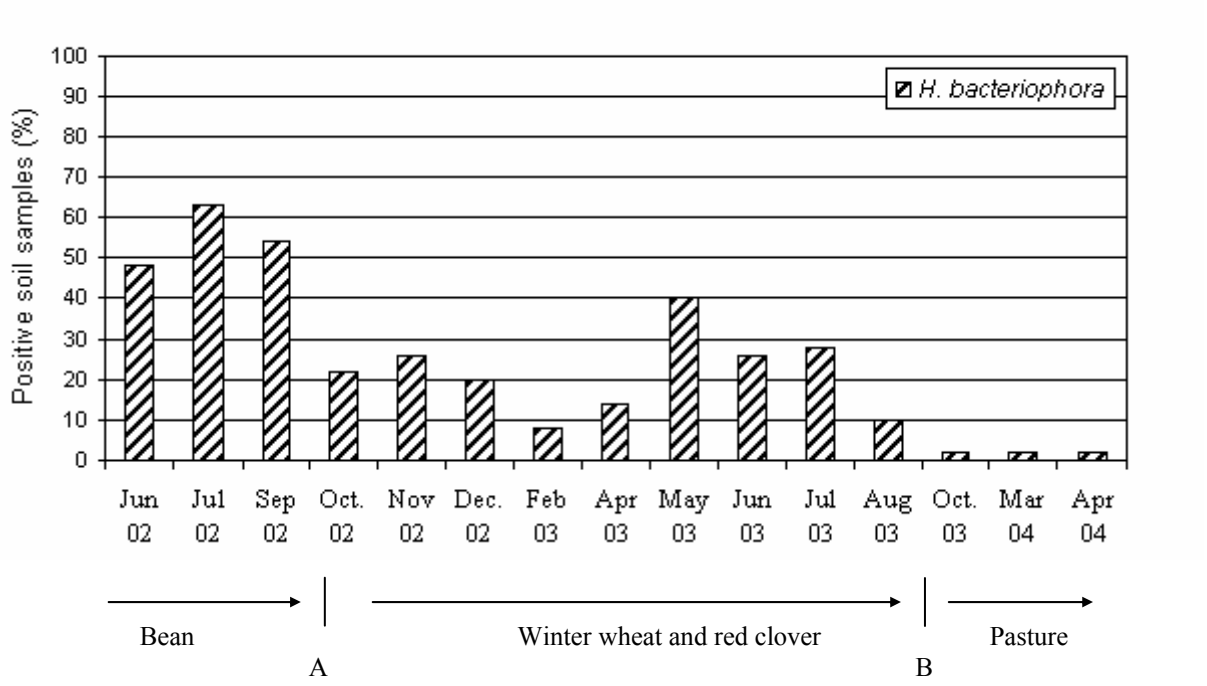

Figure 14. Percentage of soil samples (50 per sampling date and field) positive for *H. bacteriophora* released on 11.06.02 in beans at the Lindhof. Samples taken on 05.-08.2004 were negative. The horizontal arrows indicate crops in the field. The capital letters indicate tillage. A: The field was tilled with disc harrow and then ploughed and drilled; B: Field was ploughed.

Figure 14 summarized results obtained with a release of *H. bacteriophora* in bean. The longest persistence of 23 months was recorded in this field. After application 45.7 ±6.8 IJs cm^{-2} were recovered from the Petri dishes (Tab. 6). The number of the positive soil samples decreased in autumn and in winter, but increased again after February 2003. The number of positive samples decreased again after September 2003. In both cases the decline is occurring after intensive tillage.

During the sampling, larvae of the bean weevil *S. lineatus*, a common pest in beans, were frequently found in the field. The insect pest was isolated from this field mainly during June and July 2002. Most of the isolated larvae were in the 2^{nd} or last instar. Before the application, 73 non-infected larvae were detected. A month after application of *H. bacteriophora*, 58 insect larvae were found in the soil samples of July 2002. Of these 14 were infected with *H. bacteriophora* (24%). In the control samples from the site of the field without a nematode treatment, 62 larvae were found in 50 soil samples, but none were infected. The following

months, no larvae were isolated. The relatively long persistence of *H. bacteriophora* was probably caused by the presence of this suitable host insect. Although no insects were found in 2003, the under-sowing with clover might have maintained a small population of this insect thus providing prey for the released nematode population.

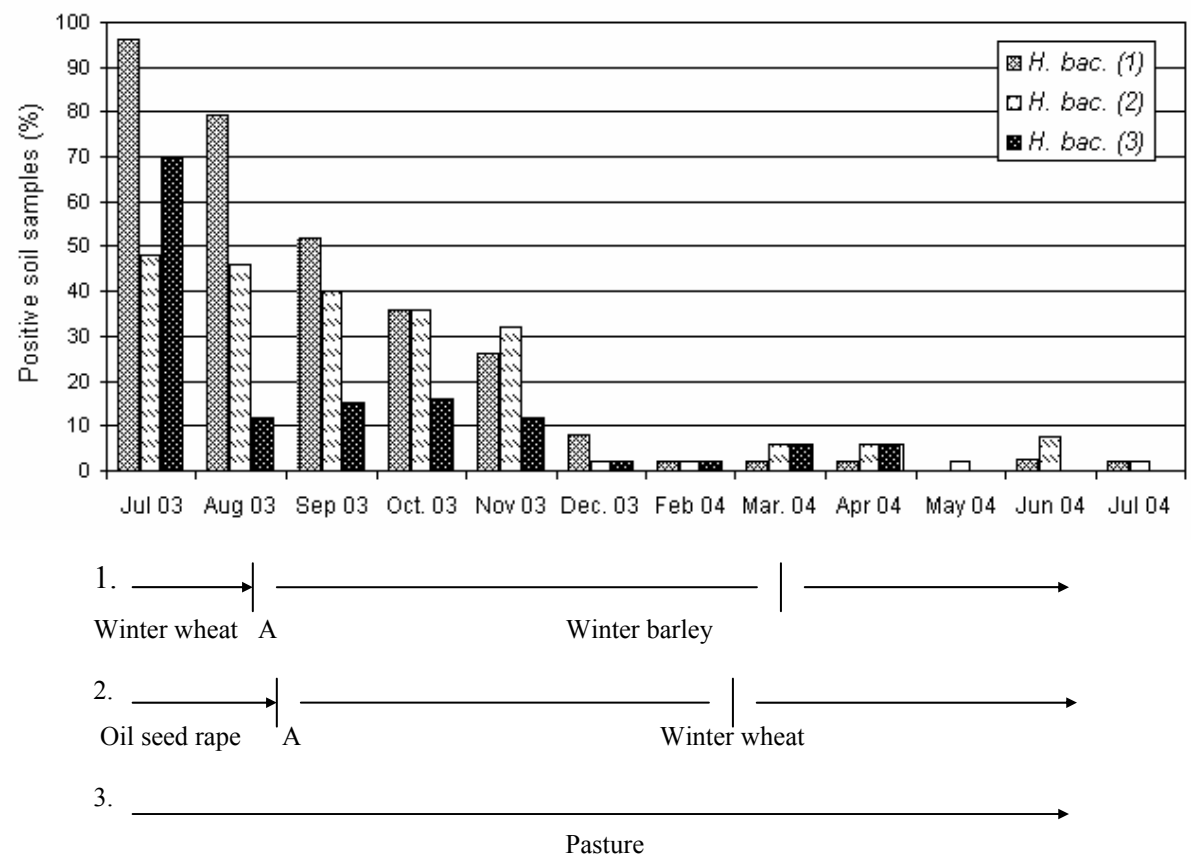

Figure 15. Percentage of soil samples (50 per sampling date and field) positive for *H. bacteriophora* released on 19.06.03 in winter wheat (1), oil seed rape (2) and pasture (3) on the farm in Rastorfer Passau. All samples taken on 08.2004 were negative. The horizontal arrows indicate crop in the fields. The capital letters indicate tillage in the fields. A: Fields were ploughed

Results obtained from fields in Rastorfer Passau (Fig. 15) indicate that the crop can have an influence on the long-term persistence. While the nematode population applied in winter wheat (*H. bacteriophora 1*) and oil seed rape (*H. bacteriophora 2*) persisted until July 2004, the population applied in pasture (*H. bacteriophora 3*) was recovered only until April 2004. In pasture the number of positive samples decline more rapidly than in the other fields. Although the number of nematodes reaching the soil after application was only 2.3 ± 1.9 in oil seed rape (Tab. 6), the number of positive soil samples immediately after application was relatively high (almost 50%). In pasture 41.8 ± 11.2 IJs cm^{-2} and in wheat only 26.2 ± 9.0 IJs cm^{-2} were counted. However, the number of positive soil samples immediately after application was higher in wheat. Even tough no *H. bacteriophora* was found after April 2004 in pasture, *H.*

bacteriophora was still isolated in wheat and oil seed rape during this time. The highest number of positive samples was recorded from winter wheat. No insects were found in the samples, why one can only speculate what the potential host might have been.

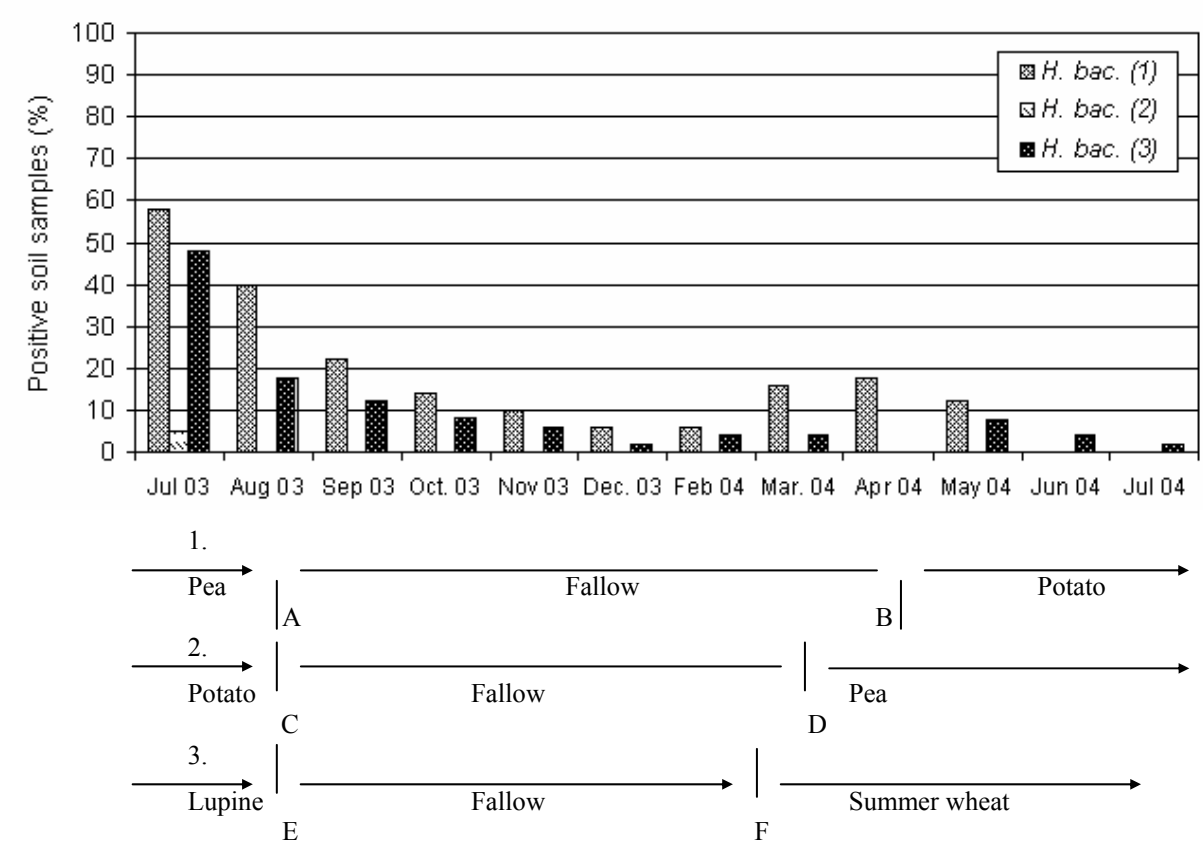

Figure 16. Percentage of soil samples (50 per sampling date and field) positive for *H. bacteriophora* released on 08.07.03 in pea (1), potato (2) and lupine (3) on the Lindhof. All samples taken on 08.2004 were negative. The horizontal arrows indicate crops in the fields. The capital letters indicate tillage in the fields. A: Disc harrow and then was ploughed and drilled; B: Field treated twice with disc harrow and then ploughed and stones removed with a machine; C: Harvest of potatoes; D: Harrow; E: Disc harrow; F: Ploughed.

Figure 16 summarizes results obtained from a release in peas, potatoes and lupine. Again, there are strong indications that tillage negatively effected the nematode population. Potato seem to be unsuitable for establishment of *H. bacteriophora*, as no positive soil sample was detected a months after application. Although the number of IJs found in Petri dishes was considerably high with 42.6 ± 5.3 IJs cm^{-2}, the nematode were not able to establish. The number of IJs reaching the soil in peas (1) and lupine (3) was 23.6 ± 6.7 and 3.0 ± 1.8 IJs cm^{-2} respectively, thus much lower than in potatoes, but nematodes were able to establish and persist. After February 2004, *H. bacteriophora* in peas increased slightly, but after May 2004 *H. bacteriophora* was not detected any more.

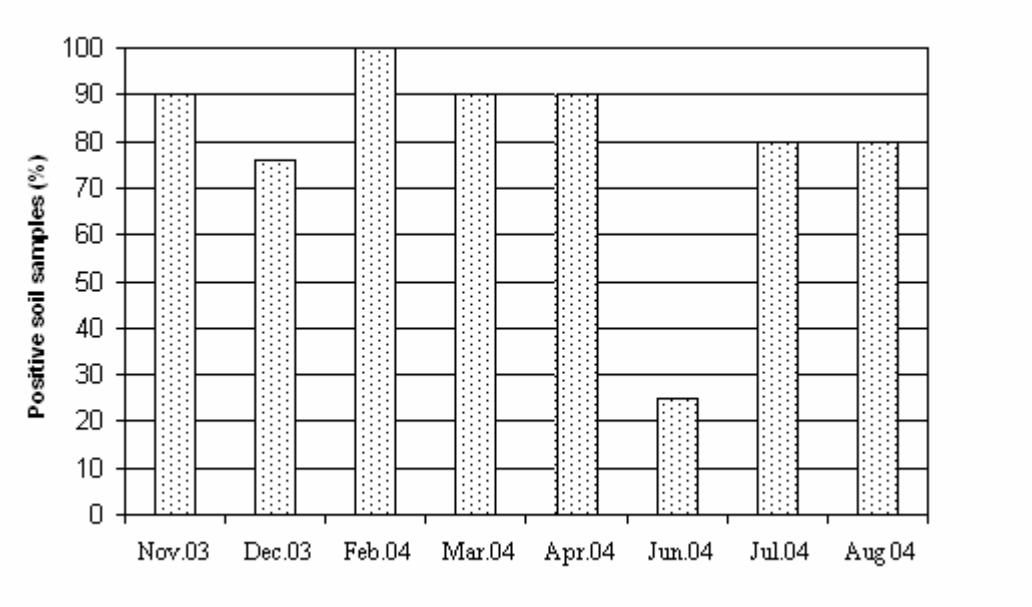

Figure 17. Percentage of soil samples (30 per sampling date and field) positive for *S. feltiae,* released on 11.11.03 in oil seed rape in Rastorfer Passau. This field was heavily infected with 3rd instar larvae of the cabbage root fly *D. radicum* during application. No crop rotated in this field during the sampling period.

The establishment and persistence of a *S. feltiae* population is documented in Fig. 17. Although the soil temperature (5 °C) during the application on November 11, 2003 was relatively lower, *S. feltiae* established well and persisted over the total length of the monitoring until August 2004. No samples we taken after that date. The level of the positive soil samples was remarkably high compared to data recorded from all other fields. The percentage even increased to 100% in February 2004 3 months after application. Sampling in February 2004 was done after a period of relatively high temperature reaching 14 °C, which might have increased dispersal of EPNs in the soil. During the sampling in this field the lowest percentage of positive soil samples was 76% in December 2003, except that in June 2004 the percentage decreased to 25%. This decrease might be related with the use of an insecticide against the rapeseed blossom beetle *Meligetis aeneus* F. (Coleoptera: Nitidulidae). However, after this month, the percentage of the positive soil sample increased again up to 80% (Fig. 17). Since the 2nd and 3rd instars larvae of the cabbage root fly were present in the field, the high number of positive soil samples might have been related with the presence of this and other potential host insects. In November 2003, the number of cabbage root fly larvae recorded in the field was 2.26 ± 0.8 per plant and no pupae were found in the soil surrounding the plants.

3.4. Persistence in laboratory experiments

The experiments were carried out at 8, 15 and 25 °C in different soils. Nematode populations were monitored by the *G. mellonella* baiting method and counted under the microscope after dissection of the infected *G. mellonella* larvae (2.5.).

Experiment I: In the experiments pots were filled with soil and the soil was treated with *S. feltiae* and *H. bacteriophora* at a dose of 50 IJs cm^{-2}. The treated pots were kept at 8 °C and sampled for 12 weeks.

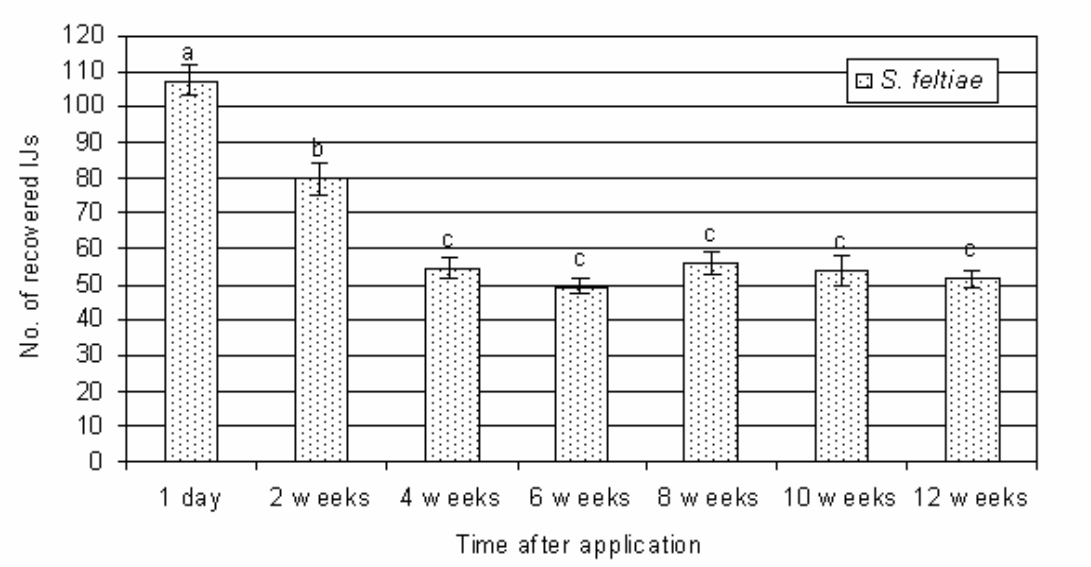

Figure 18. Mean (±SE) numbers of *S. feltiae* recovered over a period of 12 weeks after application from soil stored at 8 °C. Bars with different letters are significantly different. Data were analysed by analyse of variance ANOVA at $P < 0.05$ and the least significant test (LSD) for testing pair wise differences ($F=39.4847$; $df= 6, 133$; $p<0.0001$).

Theoretically, each sample should have contained approximately 150 IJs. In Fig. 18 the mean number of recovered IJs by the baiting method is shown. After one day little less than 110 IJs were recovered (> 70%). In this experiment the half-life time of the nematode population could not be calculated since the number of recovered IJs during 12 weeks did not differ significantly ($R = 0.2$; Variance explained =3.9%, Statistica, 1991). Thus the population decrease recorded immediately after application was more pronounced than any following changes in the population densities in the following period, although the differences in the first two weeks were statistically significant (Fig. 18).

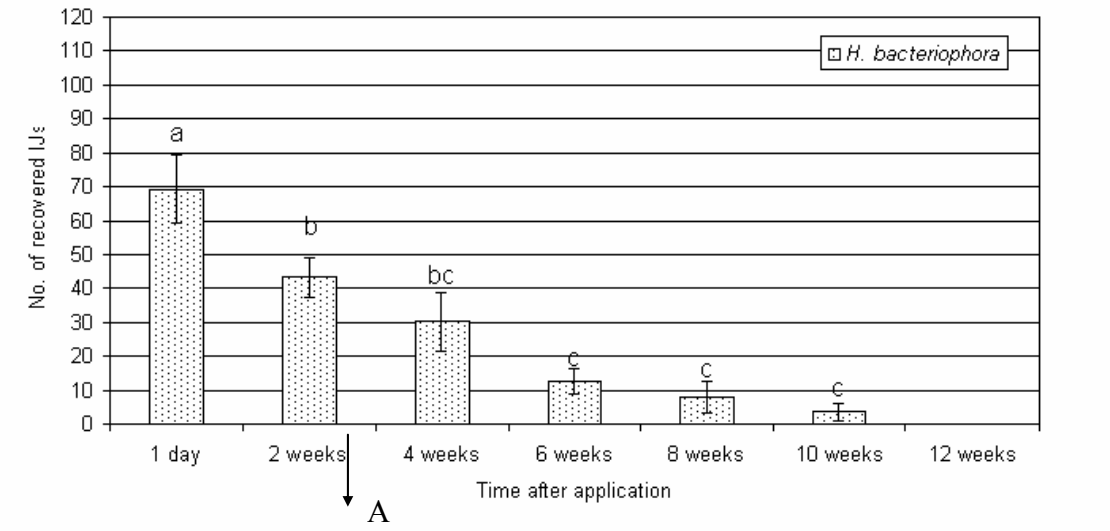

Figure 19. Mean (±SE) numbers of *H. bacteriophora* recovered over a period of 12 weeks after application from soil stored at 8 °C. The arrow (A) indicates the half-life time of *H. bacteriophora* (LT_{50} = 24.8 days according the Probit analysis). The initial number used for this calculation was the number recorded one day after application. Bars with different letters are significantly different. Data were analysed by analyse of variance ANOVA at $P < 0.05$ and the least significant test (LSD) for testing pair wise differences ((F=18.0292; d f=6, 133; p<0.0001).

The results obtained with *H. bacteriophora* are presented in Fig. 19. After the 10th week no nematodes were recovered from the soil. Considering the theoretical amount of 150 IJs in one sample the losses 1 day after application (69.3 IJs recovered) are more than 50% and therefore much higher than with *S. feltiae*. The half-life time of *H. bacteriophora* of 24.8 days (R = 0.7; Variance explained = 48.8 % and 95% fiducial limit = 23.7 - 25.9; Statistica, 1991) was calculated according to Probit analysis (2.9.).

Experiment II: In the other experiment on persistence under controlled conditions, only *H. bacteriophora* was used at temperatures of 15 and 25 °C and the experiments were conducted in sterile and un-sterile soil collected from the Lindhof and Rastorfer Passau. The results are presented in Fig. 20 and details on statistical differences are summarized in Annex 1. No statistically significant differences in the number of recovered IJs from the soil collected at the different locations were recorded (Harder test, P = 0.05). The same results were obtained when comparing persistence in sterile or un-sterile soils and persistence at 15 and 25°C. Although significant only in one case (Lindhof, un-sterile soil after 5 weeks), the temperature seems to influence persistence. When the number of recovered IJs from soil stored at 15 and 25 °C is compared, in all cases the number is lower in samples taken from soils stored at the higher temperature. In soil from the Lindhof, this effect is more pronounced in un-sterile than in sterile soil samples. However, this could not be recorded in soil from Rastorfer Passau.

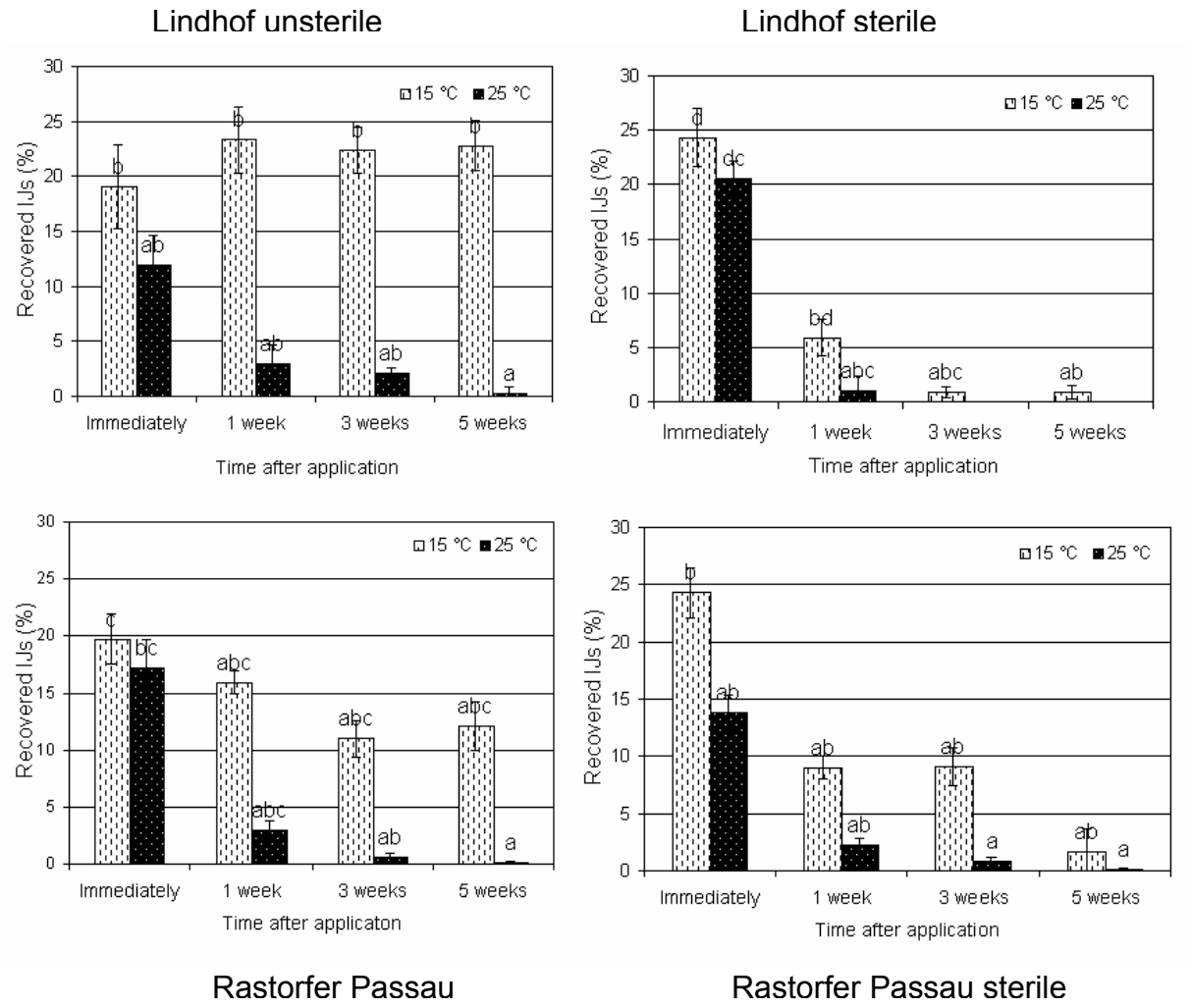

Figure 20. Mean percentage (±SE) recovered IJs of *H. bacteriophora* of an initial theoretical number of 150 IJs applied to sterile and unsterile soil samples collected from the Lindhof and Rastorfer Passau and stored at temperatures of 15 and 25°C after application of the nematodes. At 7 and 9 weeks after nematode application no nematodes were detected in any of the samples. Bars with the same are not significantly different (P = 0.05; n = 6; k = 32 and q_{kp} = 114.6 according to Harder Test).

In general, in none of the samples *H. bacteriophora* could persist for longer than 5 weeks and the decline was more pronounced at higher temperature.

3.5. Influence of application time and crop on establishment

Whether establishment and persistence might be influenced by the crop and the time of application was investigated on plots on the Lindhof and in Rastorfer Passau (2.4.2.). *H. bacteriophora* was applied to a total of eight different crops between March and July 2004. Nematodes were sprayed 2 to 4 times to each crop depending on the date of sowing (e.g. corn was not treated before May 2004) and the height of the plant (oil seed rape was not treated after April 2004 since the crop was > 2 m).

Meteorological data for the Lindhof and Rastorfer Passau are presented in Annex 2 and 3. Table 9 summarizes data on the number of IJs recorded in Petri dishes under the plants, the height of the plants at the moment of application and data recorded for soil and air temperature and relative humidity during the day of spraying and precipitation measured during the week following application.

Table 8. Pathogenicity of different batches of *H. bacteriophora* (assessed according to 2.3.4.) used in the establishment experiment.

Appl. Dates	Date of harvest	LD_{50}	95% fiducial limits for the LD_{50}	LD_{90}	95% fiducial limits for the LD_{90}
24.03.2004	15.03.2004 [a]	-	-	-	-
05.04.2004	28.03.2004	5.9-A	3.3 - 8.0	27.9-AB	19.0 - 60.1
27.04.2004	26.04.2004	9.2-A	6.7 - 12.0	26.5-A	20.3 - 40.7
11.05.2004	26.04.2004	9.2-A	6.4 -11.9	26.5-A	20.3-40.7
07.06.2004	24.05.2004	12.9-B	9.5 - 17.4	71.9-B	41.3 - 258.3
11.06.2004	24.05.2004	12.9-B	9.5 - 17.4	71.9-B	41.3 - 258.3
15.07.2004	05.07.2004	5.8-A	3.1 - 7.9	19.1-A	13.9 - 38.0

Note: [a] Bioassays not conducted. Pathogenicity tests were conducted at the time of harvest from liquid cultures. Data with the same capital are not significantly different according to Probit analysis (95% fiducial limits).

In order to exclude an influence of nematode quality, the LD_{50} and LD_{90} values of the nematode population applied was recorded. According to the results calculated by Probit analysis for the pathogenicity of the nematodes, no significant differences were detected between the different production batches except the batch which had been produced on May 24, 2004 (Tab. 8). However, the LD_{50} was still low enough to justify application in the field.

Table 9. Application dates, crop, results of nematode recovery immediately after application (appl.), recovery of EPN with Petri dishes at the moment of application, precipitation in the week after application and climatic conditions during application.

Crops and Appl. Dates	Positive soil samples (%) immediately after appl.	Height of the plants (cm)	Appl. Dosage (IJ/cm²)	Total precipitation in week after appl. (mm)	Soil temperature (°C)	Air temperature (°C)	Relative air humidity (%)
Pasture, 24.3.04	15.7	9.4 ±2.7	62.0 ±5.5	-	-	-	-
Pasture, 27.4.04	77.5	17.4 ±3.4	50.9 ±6.2	5.2	10	10	91
Pasture, 01.6.04	45	8.4 ±2.2	56.8 ±5.3	2.8	13.2	14.6	76
Pasture, 02.7.04	90	9.1 ±2.2	38.0 ±4.1	27.7	14.3	12.3	90.7
Wheat, 24.3.04	28	10.7 ±2.6	41.6 ±6.9	-	-	-	-
Wheat, 27.4.04	100	20.9 ±2.9	22.1 ±2.1	5.2	10	10	91
Wheat, 01.6.04	2.5	111 ±7.9	6.1 ±1.2	2.8	13.2	14.6	76
Wheat, 02.7.04	70	109.8 ±7.1	7.7 ±2.5	27.7	14.3	12.3	90.7
Oil-seed rape, 24.3.04	26.5	19.2 ±4.2	35.1 ±5.7	-	-	-	-
Oil-seed rape, 27.4.04	97.6	70.4 ±12.4	42.0 ±6.3	5.2	10	10	91
Corn, 01.6.04	30	7.3 ±1.8	46.9 ±3.8	2.8	13.2	14.6	76
Corn, 02.7.04	100	61.3 ±3.2	39.7 ±4.9	27.7	14.3	12.3	90.7
Clover, 05.4.04	72.5	10.0 ±2.6	60.6 ±6.7	8.4	6.2	6	90.1
Clover, 11.5.04	47.5	50.1 ±6.4	12.7 ±2.3	8.6	12.9	10.8	95.4
Clover, 07.6.04	15	17.6 ±3.8	29.6 ±4.4	4.8	17.3	18.4	77.2
Clover, 15.7.04	88	10.7 ±1.7	54.9 ±5.7	18.5	15.7	16.2	79.8
Barley, 05.4.04	80	10.2 ±2.6	62.7 ±4.7	8.4	6.2	6	90.1
Barley, 11.5.04	90	54.0 ±6.5	38.3 ±6.1	8.6	12.9	10.8	95.4
Barley, 07.6.04	65	112.8 ±8.4	15.2 ±2.5	4.8	17.3	18.4	77.2
Barley, 15.7.04	97.4	103.7 ±7.05	44.9 ±4.0	18.5	15.7	16.2	79.8
Pea, 11.5.04	100	3.8 ±1.03	40.4 ±2.1	8.6	12.9	10.8	95.4
Pea, 07.6.04	62.5	33.2 ±4.6	37.2 ±18.4	4.8	17.3	18.4	77.2
Pea, 15.7.04	87.8	60 ±4.2	9.9 ±4.0	18.5	15.7	16.2	79.8
Potato, 07.6.04	12.5	26.9 ±3.5	31.9 ±5.3	4.8	17.3	18.4	77.2
Potato, 15.7.04	87.5	35.7 ±3.9	13.5 ±4.9	18.5	15.7	16.2	79.8

An influence of the application was not tested, as previous results had indicated that the sprayer hardly affected the survival and pathogenicity of the nematodes (Tab. 6).

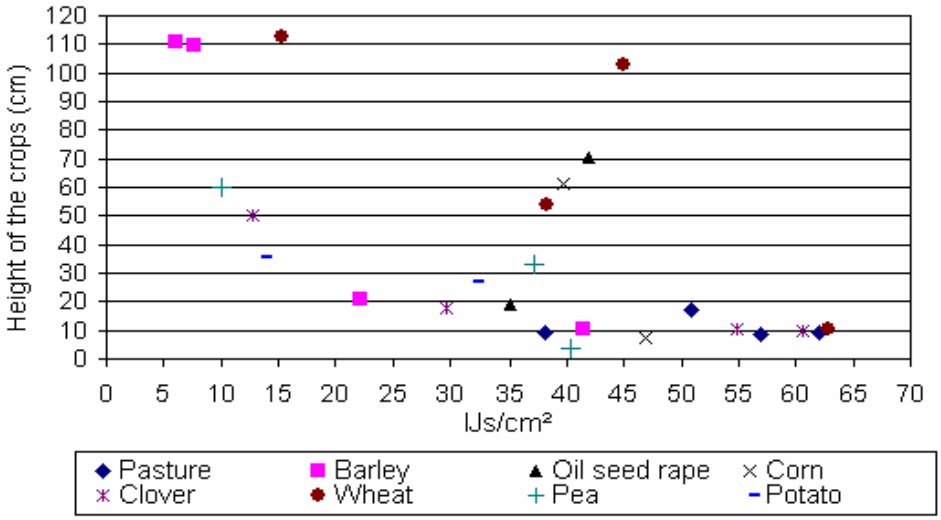

Figure 21. Relationship between height of the crop at the time of application and number of IJs per cm² recovered from Petri dishes below the plants at the time of application.

A negative correlation ($r = -0.62$, $P = 0.001$) was achieved comparing the heights of the crops and the IJs per cm² detected in the Petri dishes below the plants (Fig. 21). This relation cannot be generalized as the plant surface (e.g. waxy cuticle) can have a significant impact on the run-off of the EPN suspension. However, the higher the index for the leaf area, the more EPN can possibly remain on the plant canopy and will not immediately reach the soil. An exception from this consideration seems to be wheat (Fig. 21), in which a low and high number of IJs were recovered on considerably high plants.

In another step the data on the IJs recovered under the canopy were presented in relation to the crop and time of application (Fig. 22). In general, one would expect that with increasing time the number recovered below the plants would decrease. Pasture, as always grazed by cows and thus lower than other crops, should be an exception. In fact, in pasture the number of recovered IJs was usually higher than in other crops.

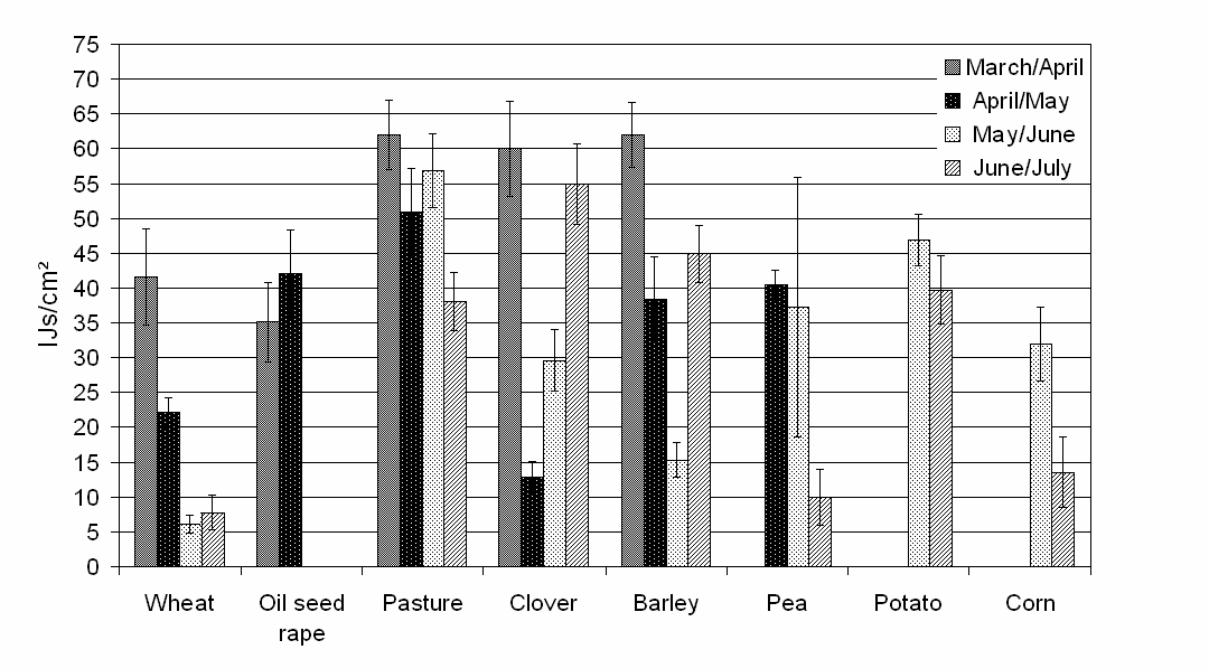

Figure 22. Mean (±SE) numbers of IJs recovered immediately after spraying from Petri dishes placed below the plant canopy in the months March, April, May, June and July.

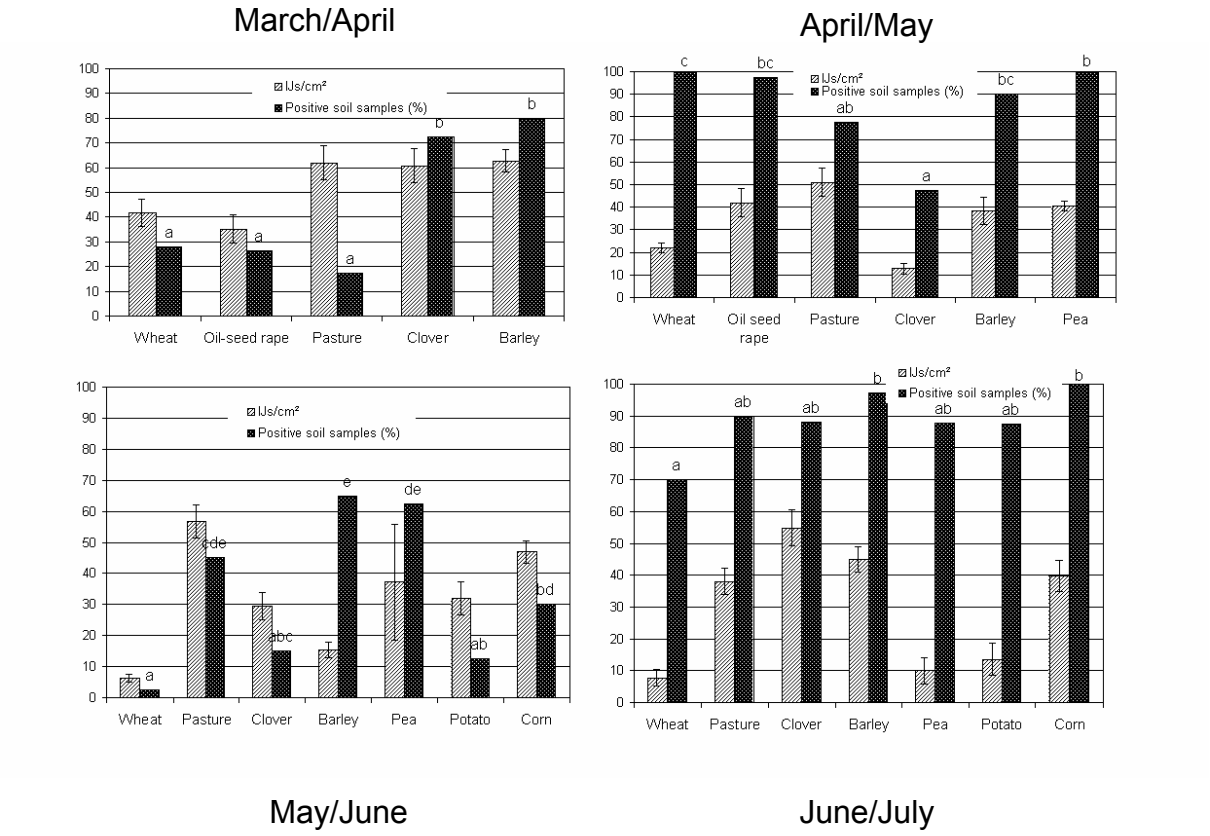

Figure 23. Mean (±SE) numbers of IJs recovered immediately after spraying from Petri dishes (n=10) placed below the plant canopy in different crops and number of positive soil samples (n = 40) in the months March (x = 23.4 ± 6.7%), April (x = 85.5 ± 12.4%), May (x = 79.2 ± 27.9%), June (x = 33.6 ± 24.9%) and July (x = 88.7 ± 9.6%) obtained immediately after application. Data for positive soil samples followed by the same letter are not significantly different according to the Chi² test (P = 0.05).

In Fig. 23 the data on the number of IJs encountered in the Petri dishes are jointly presented with the percentage of positive soil samples recorded immediately after spraying. There is no correlation between the number of IJs per cm² recovered from dishes and percentage of positive soil samples ($r = 0.07$; $p = 0.75$). Usually one would expect that a closer correlation would be detected, as the number applied EPN should also be found in the soil samples. The fact that this correlation is not existing indicates that although many nematodes might remain in the canopy after spraying they must not necessarily remain on the plant but get washed into the soil with rain.

In general, the lowest amount of positive soils were detected in March/April and May/June, while samples collected in April/May and June/July more often contained nematodes of the released species *H. bacteriophora*. During all applications, 100% positive soil samples were detected only in three applications. They were in wheat and pea in April and in corn in June. However, the number of the IJs in the dishes was variable with 40.4 ± 2.1 and 39.7 ± 5.0 IJs per cm² in peas and corn, respectively and 22.1 ± 2.1 IJs per m² in wheat. The lowest number (6.1 IJs per cm²) was recorded in dishes placed under wheat in May, whereas positive soil samples were 70% immediately after application (Tab. 9). All these results indicate that the amount of IJs found in the dishes has no effect on the establishment.

In Fig. 24 the results on establishment (positive records immediately after application) are presented for the different crops. Significant differences were detected over time in the different crops. However, no reasonable explanation is available why during one time the establishment is high and in the following moth it is low, e.g., the establishment in wheat in April/May was high (100%) and in the following month is did not surpass 2.5%. As already mentioned, the impact of the plant canopy on establishment can be neglected. So other reasons must be discussed.

The lowest establishment was recorded in the month June (33.6 ± 24.9%), whereas results obtained with applications in July were much more promising (88.7 ± 9.6%) and the variance was lower. One possible explanation is that weather conditions might have had an influence on these results.

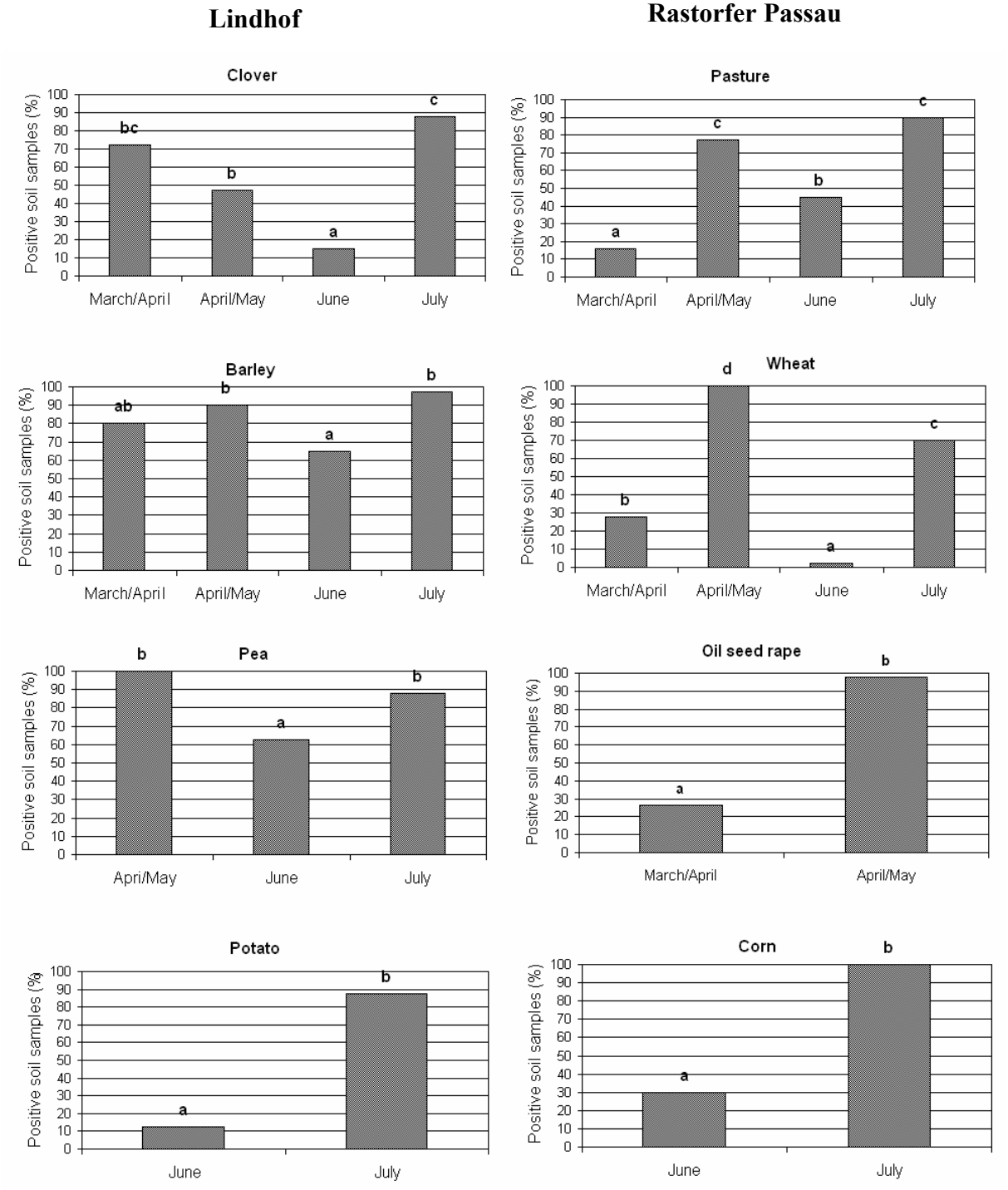

Figure 24. Establishment of *H. bacteriophora* recorded as positive soil sample obtained immediately after application in different crops and application dates. Bars in one graph with the same letters are not significantly different (Chi² Test, P = 0.05).

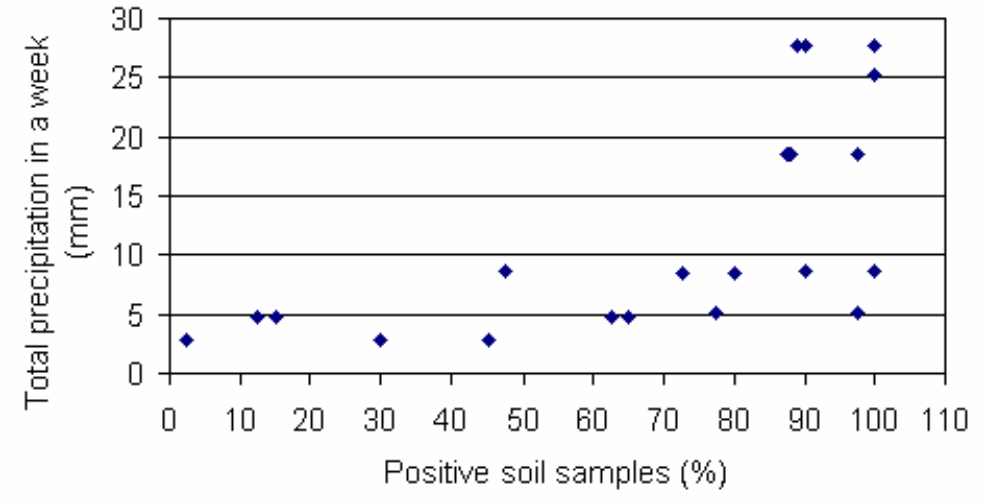

Figure 25. Relation between numbers of the positive soil samples and precipitation recorded in the week immediately after application (Correlation r = 0.64; P = 0.001).

In Fig. 25 the number of positive samples is plotted against the precipitation (mm) recorded during the week after application. The correlation coefficient r = 0.64 (P = 0.001) is significant with a probability of 95%. For the relative humidity a correlation of r = 0.57 (P = 0.005), for the radiation r = - 0.44 (P = 0.04) and for the air temperature r = - 0.41 (P = 0.06) were calculated summarizing these factors as the most influencing on nematode establishment. Other factors, like soil temperature, height of crop, and number of nematodes in the dishes had no significant influence on establishment. All factors influencing establishment indicate that nematode survival is a matter of humidity at and after application.

3.6. Persistence of *Heterorhabditis bacteriophora*

Besides the establishment of the nematode *H. bacteriophora* in different crops, the persistence of the released nematodes, respectively, the record of their offspring was observed in the fields treated in 2004 until August 2004. The longest persistence was recorded in clover for three moths (until July), but after this time no nematodes were detected in this field. After the first application, no positive samples were found in pasture for two application dates (April and June) and in corn, potatoes and peas in June (Fig. 26). In general, the number of positive soil samples was significantly reduced in 16 out of 18 cases and the reduction was usually between 80 and 100%. Only in two cases the decrease in positive soil samples was less than 60%, in winter barley and wheat treated in April and May, respectively. In most crops (10 applications out of 18) the nematodes persisted at very low frequency of 5% and less in the second month after application. In another 3, the percentage of positive samples was less than

10%. In wheat, the number was 12.5% (April 5 application) and 37.5% (May 11 application). In winter barley (April 27 application) the highest percentage of soil samples on the second evaluation was recorded with 50% and it remained high with 42.5% a month later. In all other cases the number did not surpass 10% in the third evaluation. In general, the persistence was low in almost all applications.

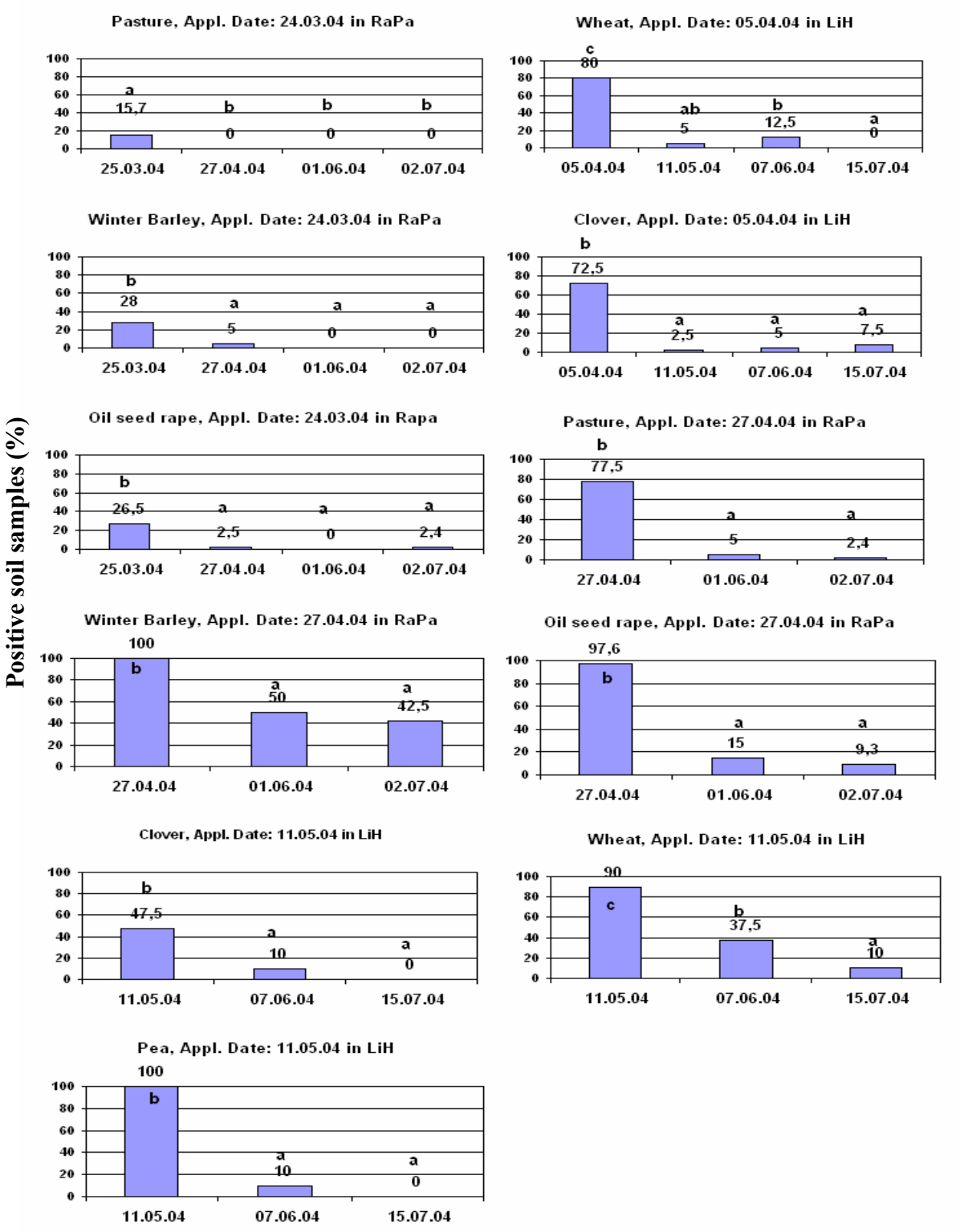

Figure 26. Persistence *of H. bacteriophora* in different crops and at different application dates. Columns with the same letter in one graph are not significantly different (Chi² Test, P = 0.05).

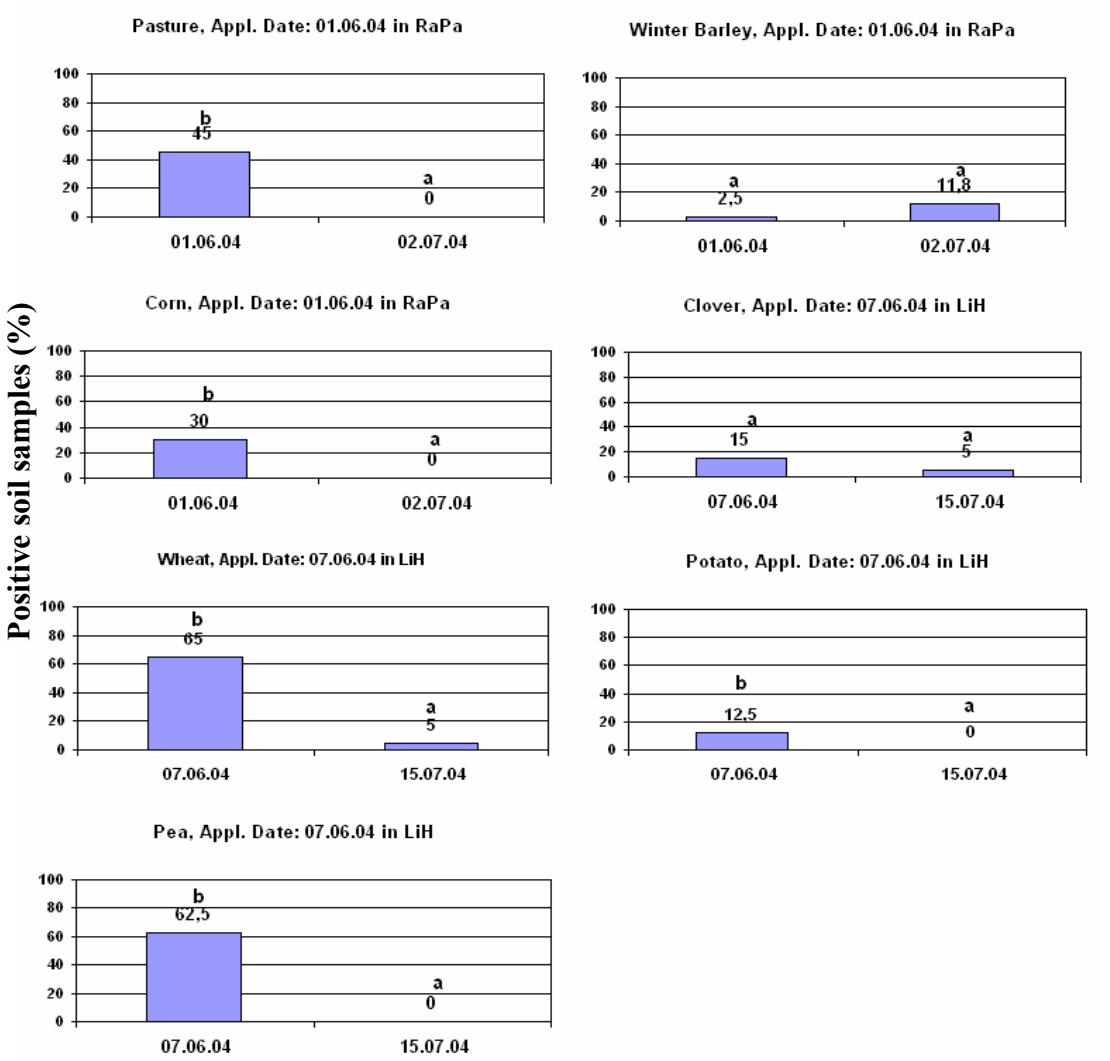

Figure 26 (Conti.). Persistence *of H. bacteriophora* in different crops and at different application dates. Columns with the same letter in one graph are not significantly different (Chi² Test, P = 0.05).

3.7. Characterisation of re-isolated *Heterorhabditis bacteriophora* population

A population of *H. bacteriophora*, which had been released on the Lindhof in beans in June 2002, was re-isolated in June 2003 and subjected to efficacy tests at different temperature, reproduction potential and persistence under laboratory conditions. Results were compared with those of a population received from e-nema GmbH produced in liquid culture. The nematode was recovered from the soil with the *G. mellonella* baiting method. Nematodes from liquid culture were propagated once in the *G. mellonella*.

The data in Fig. 27 indicate that differences in infectivity of *H. bacteriophora* originating from *in-vitro* culture and re-isolated nematodes can be detected particularly when low

nematode concentrations are used against *T. molitor* larvae. At low temperature the difference in infectivity is non statistically different. Significant differences were detected at a concentration of < 120 IJs at 18 °C and at < 60 IJs per larva at 24 °C. Thus re-isolated nematodes were more effective than those, which had been continuously produced, in *in vitro* culture ($F = 70.9$, $df = 5, 54$, $p<0.00001$ for the dose of 5 IJs; $F = 117.3$, $df = 5, 54$, $p<0.00001$ for the dose of 10 IJs and $F = 188.1$, $df = 5, 54$, $p<0.001$ for the dose of 30 IJs). In control treatments, mortality was between 0 and 8%.

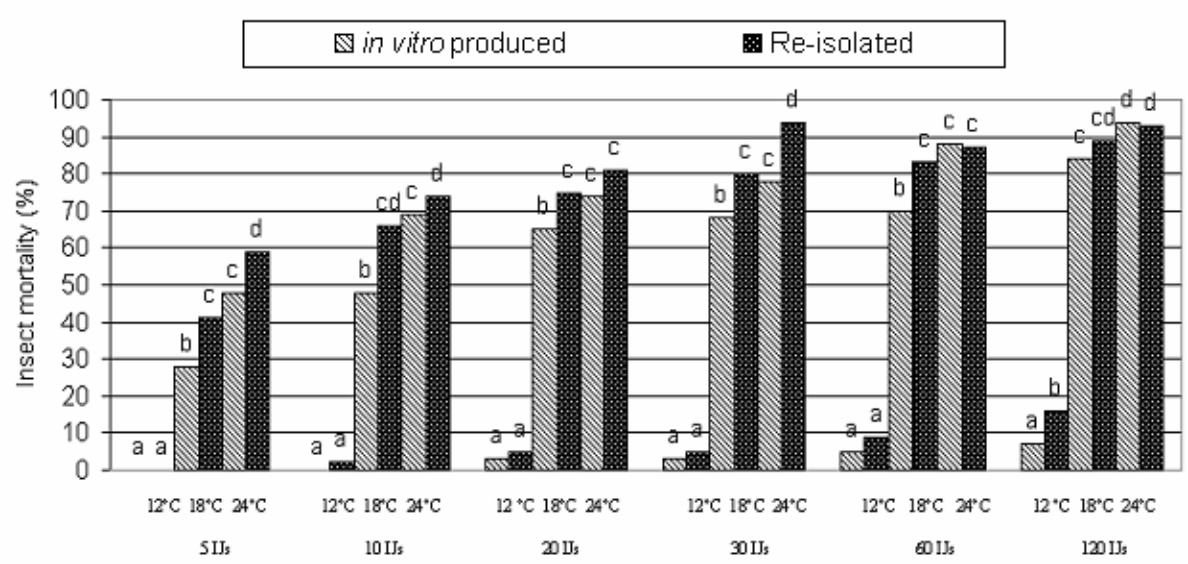

Figure 27. Mortality of *T. molitor* larvae (%) exposed for 5 days to *in vitro* produced and re-isolated *H. bacteriophora* at doses of 5, 10, 20, 30, 60 and 120 IJs at 12, 18 and 24 °C. Columns with the same letter are not statistically different for one dose. The data were analysed for variance (F-tests, $P<0.05$) and subjected to the least significant-different test (LSD) for testing pair wise differences between treatments.

Results on the reproduction of IJs are presented in Fig. 28. The re-isolated *H. bacteriophora* was more productive ($F = 31.4$; $df = 5, 54$; $p<0.00001$) except that the differences at a dose of 50 IJs were not statistically different between the two groups.

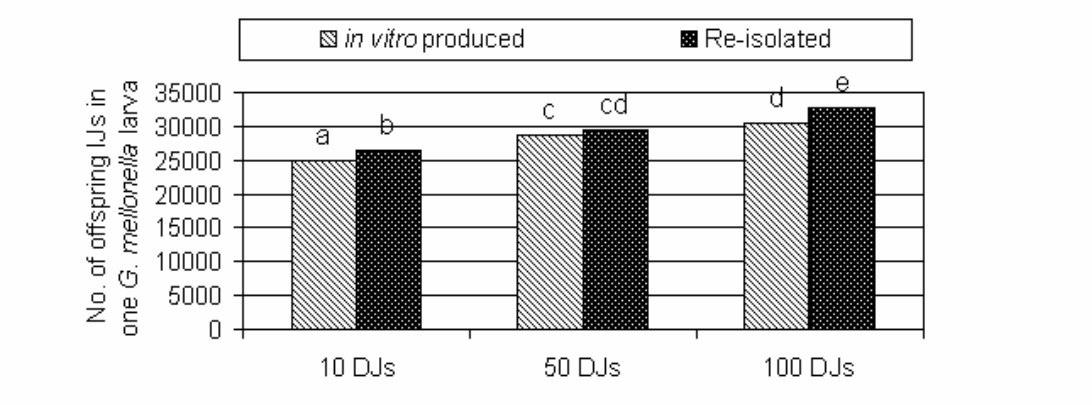

Figure 28. Number of IJs recovered from one *G. mellonella* last instar larva inoculated with *in* vitro produced and re-isolated *H. bacteriophora* at different doses. Data followed by the same letters are not significantly different from each other at p<0.05. The data were analyzed for variance (F-tests, P<0.05) and then subjected to the least significant-different test (LSD) for testing pair wise differences between treatments.

In Fig. 29 the results on the persistence are presented, indicating that the number of recovered *H. bacteriophora* IJs was usually higher in tests with the re-isolated population. The tests were replicated until no IJs were detected. Except for the 4th and 12th week, statistically differences between the two populations were detected (F = 117.7; df = 13, 266; p = 0.001).

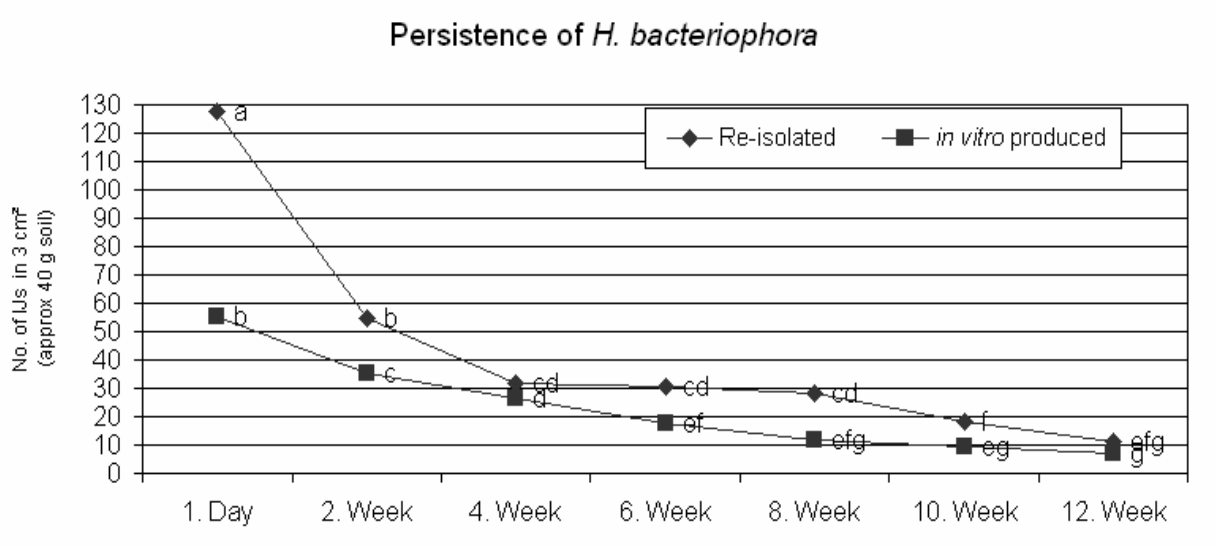

Figure 29. Numbers of recovered IJs of *in vitro* produced and the re-isolated *H. bacteriophora* from the soil which had been stored at 16 °C. Data points with the same letters are not significantly different from each other at p<0.05. The data were analysed for variance (F-tests, P<0.05) and then subjected to the least significant-different test (LSD) for testing pair wise differences between treatments.

3.8. Persistence and efficacy of *Heterorhabditis bacteriophora* against *Otiorhynchus sulcatus*

Roots of young strawberry plants were dipped into a *H. bacteriophora* solution containing the sticker CMC (Carboxymethylcellulose) at 0.5 %. The roots system of each Frigo plant took up approximately 4.4 ± 1.2 g of this suspension. Each plant thus received between 3,360 and 11,840 IJs (mean 7,020 ± 1,990 IJs per plant). These plants were then inoculated with 30 eggs of *O. sulcatus* and again inoculated with 20 eggs a month later and another 20 another months later. Mortality of the surviving larvae was assessed during 3 months. In Fig. 30 the corrected mortality of *O. sulcatus* is presented. Mortality varied between 92 and 95 %. The survival of inoculated eggs was approximately 20% (78, 80 and 80 % mortality recorded each month). The mortalities for each month in control pots were compared with treated pots and the data were analysed with the Mann-Whitney test at $p<0.05$. According to the results, the differences between the treatments and the controls were significantly different (U (variance) = 165.5; Z (observed value) = -3.8 for the 1st month, U = 170.9; Z = -3.8 for the 2nd month and U = 171.8; Z = -3.8 for the 3rd month). No infected *O. sulcatus* larva was detected in the first month, whereas in the following two months, 42.8 and 58.8 % infected larvae were recorded. Throughout the experiment the length of the instars was recorded. The results are shown in Fig. 31. Data presented in Fig. 32 summarize the effect of EPN on the survival of the plants. With a mean number of 14 larvae per plant mortality was considerably increasing with the age, respectively instar (Fig. 31) of the larvae. The mortality of strawberry plants in control pots was higher than in pots, which had been treated with *H. bacteriophora*. The results indicate that nematodes applied during planting of strawberries can control *O. sulcatus* larvae, although an infestation of the plant is occurring after the application of the nematodes.

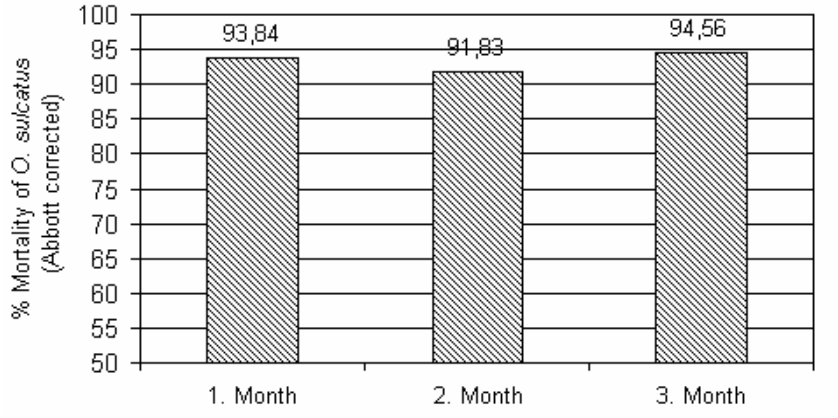

Figure 30. Abbott corrected % mortality of *O. sulcatus* caused by *H. bacteriophora* over a period of 3 months after application of the nematodes by dipping the plant roots into a nematode suspension.

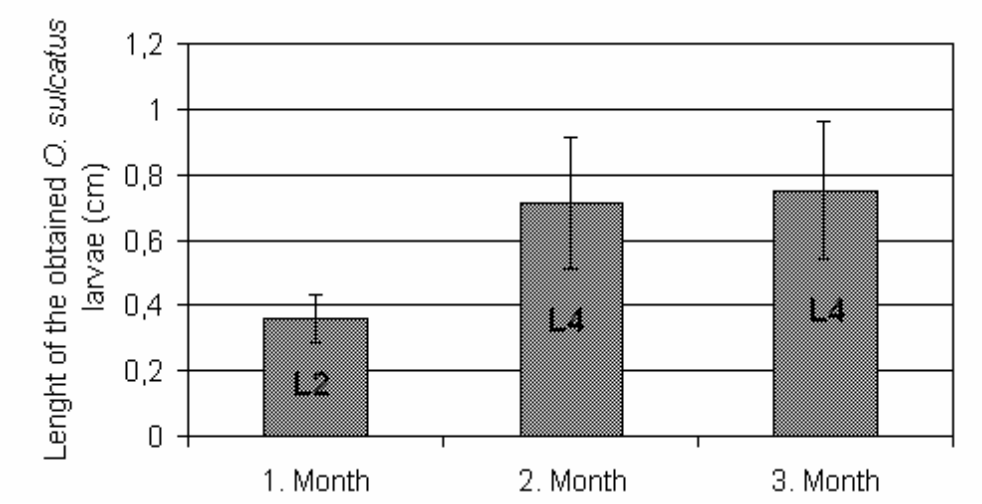

Figure 31. Development stages *of O. sulcatus* larvae observed in the experiment during 3 months. L2: Second instar larvae and L4: Fourth instar larvae.

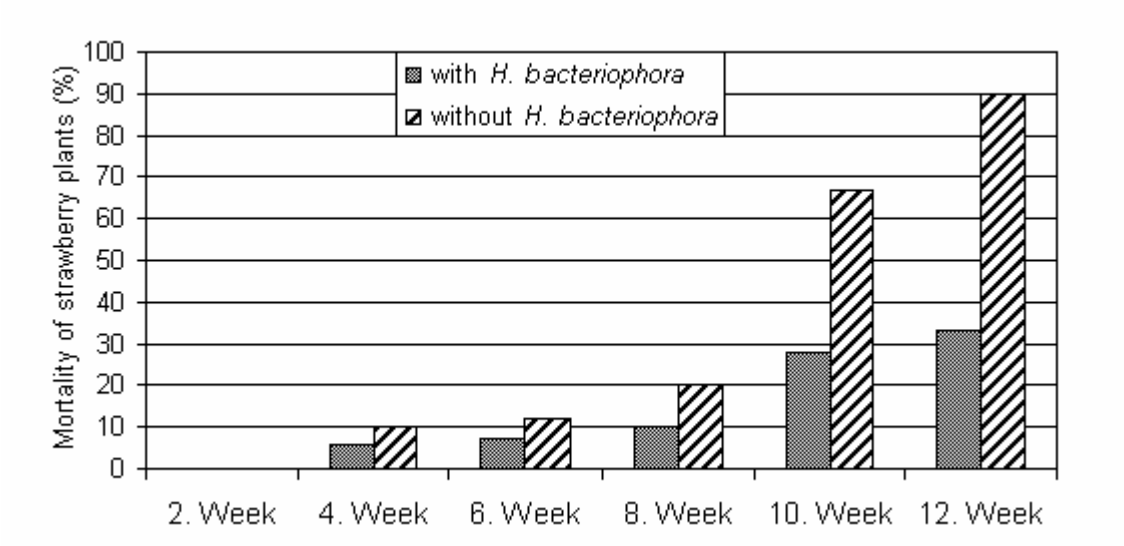

Figure 32. Percentage of dead strawberry plant caused by an infestation with a mean of 14 *O. sulcatus* larvae in treatment (with *H. bacteriophora*) and control pots (without *H. bacteriophora*) over a period of 12 weeks.

The soil was also subjected to tests evaluating EPN persistence in the pots with or without *O. sulcatus* larvae. The persistence was tested subjecting soil to *T. molitor* larvae and assessing their mortality. The results indicate that *H. bacteriophora* persisted more successful in the pots, which had been inoculated with *O. sulcatus* larvae than in the pots that had no insects (Fig. 33). Pathogenicity of *H. bacteriophora* to larvae of *T. molitor* was significantly higher in pots containing larvae of *O. sulcatus,* except in week 12 and 1 day after application ($F = 74.3$, $df = 13, 56$; $p<0.00001$).

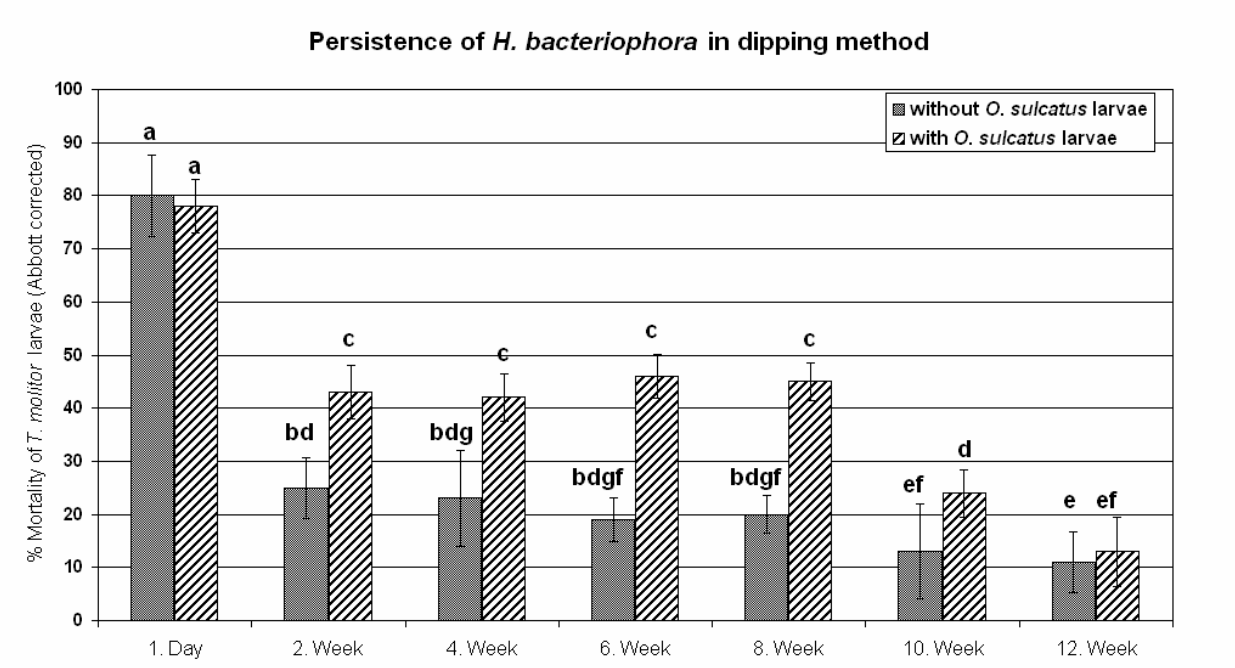

Figure 33. Persistence of *H. bacteriophora* applied by dipping the roots of strawberry plants and measured as mortality of to *T. molitor* larvae. Pots were with and without *O. sulcatus* larvae and soil samples subjected to *T. molitor* larvae over a period of 12 weeks. Columns with the same letter are not statistically different. Data were analyzed by the F-tests ($p<0.05$) and subjected to the least significant-different test (LSD) for testing pair wise differences between treatments. Error bars represent standard error of the means.

3.9.Persistence and efficacy of *Steinernema feltiae* against *Delia radicum*

In the past few years, *D. radicum* has become a major problem in oil seed rape. Therefore, experiments were conducted to control *D. radicum* with *S. feltiae*. Damage by the cabbage root fly is apparent on older leaves of infested oil seed rape plants. They turn reddish to dark purple and the roots of the plants are often cut in approximately half of the plants. In general, the infected plants are weak and smaller than healthy oil seed rape plants. Pot experiments were conducted to test the efficacy of *S. feltiae* against larvae of the cabbage root fly. No pupae were detected in the experiments when they were started, but with the advance of the experiment pupation increased from 36 ± 30% in week 2, 84 ± 41% in week 4 and 100 % in week 6 after starting the tests. Corrected mortality was calculated by comparing *D. radicum* in control and treatment pots. The results obtained revealed statistically significant differences according to the Mann Whitney test ($P<0.05$). The mortality of *D. radicum* hardly increased after week 4. As pupae are not susceptible to EPN, a major increase of the mortality cannot be expected with advancing pupation (Fig. 34). Similar results were observed with plant mortality (Fig. 35), which did not advance with progressing pupation. Due to the fact that the infected larvae of the insect are destroyed very soon after *S. feltiae* penetrated, very few

infected larvae were found in experimental pots. Therefore, the calculation of the mortality was based on the number of the surviving larvae in treatment and control pots.

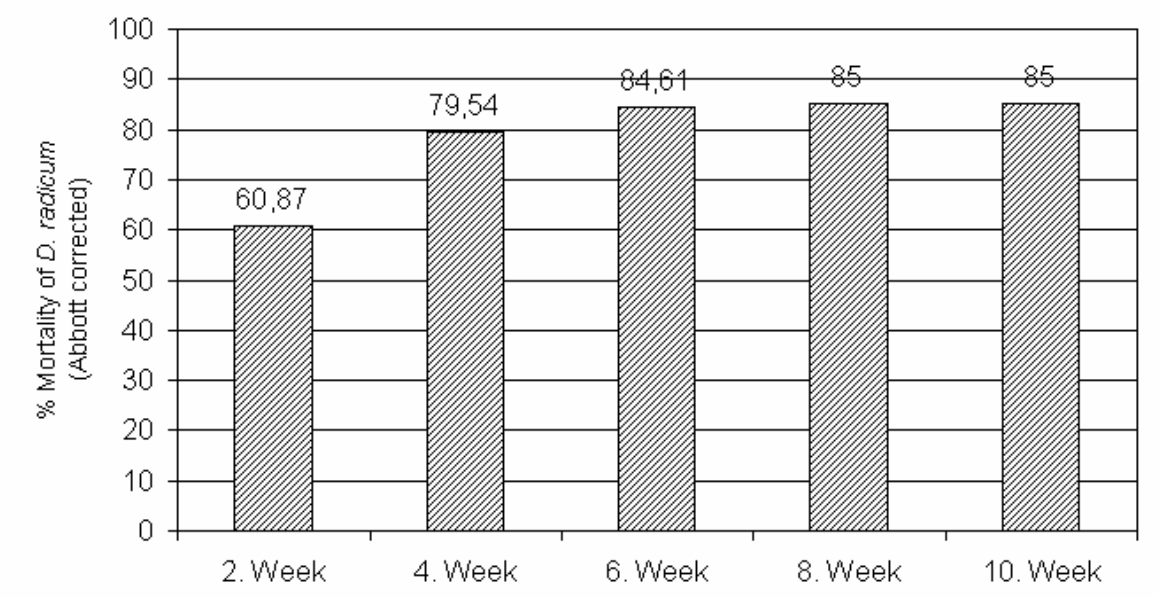

Figure 34. Abbott corrected mortality of *D. radicum* caused by *S. feltiae* in oil seed rape pots over a period of 10 weeks.

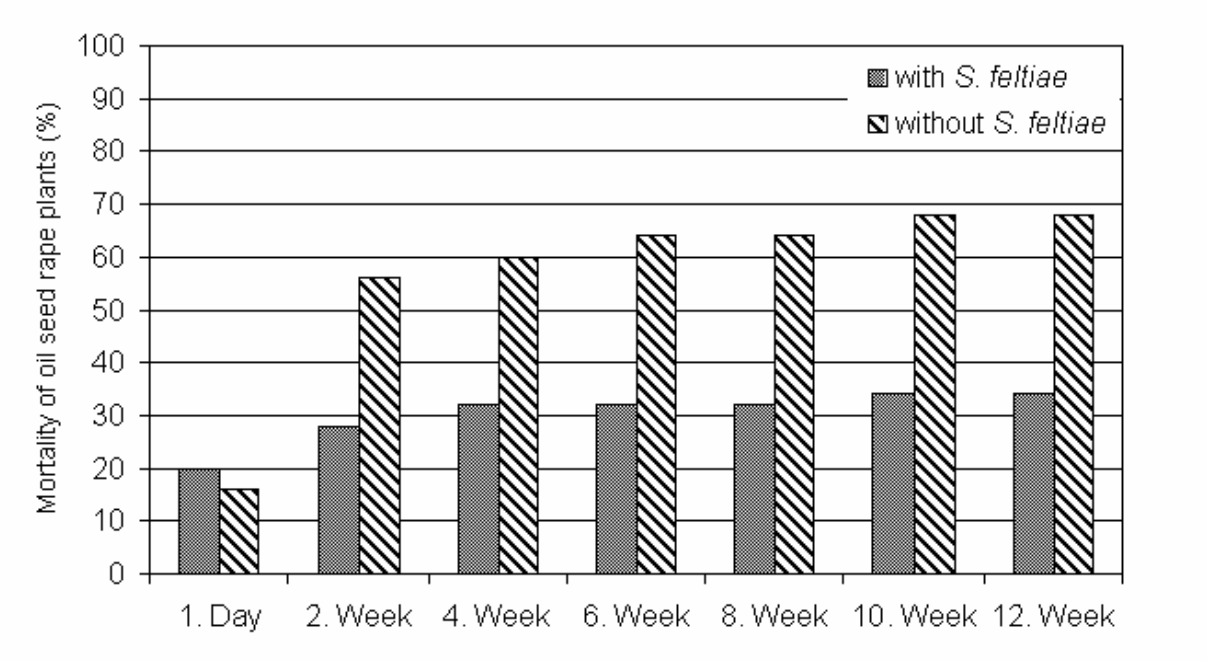

Figure 35. Mortality of oil seed rape plants caused by *D. radicum* larvae in treatment (with *S. feltiae*) and control (without *S. feltiae*) over a period of 12 weeks.

In order to detect persistence of the nematode in oil seed rape infested with the cabbage root fly *D. radicum*, pots were kept at 8 °C over a period of 12 weeks and soil samples were tested with *G. mellonella* for the presence of EPN (2.5.). In Fig. 36 the results are summarized. No

significant correlation was observed (r = 0.24, p = 0.59) between the number of positive soil samples and the number of IJs recovered from infested *G. mellonella*. A minimum of 78% and maximum of 93 % of soil samples were positive for *S. feltiae*, indicating that the nematodes persisted for very long at 8°C.

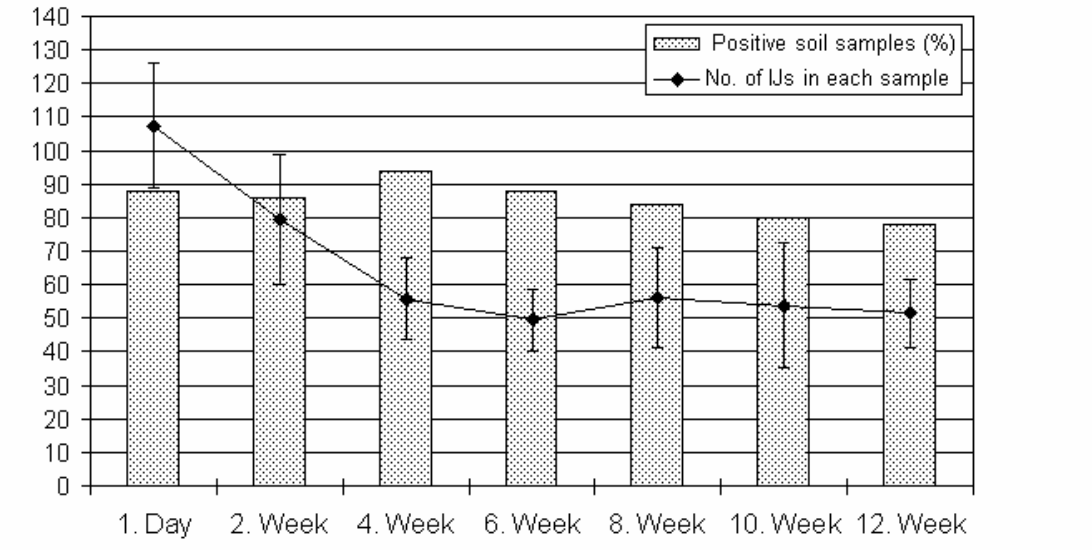

Figure 36. Percentage of soil samples positive for *S. feltiae* recorded with the *G. mellonella* baiting method and mean number (± SE) of recovered IJs from the infected insects (n=25) over a period of 12 weeks.

In an additional experiment it was investigated whether *S. feltiae* responds towards the cabbage root fly *D. radicum* or roots of oil seed rape in Y-Olfactometers. The experiments were conducted at 8 and 15 °C and replicated 3 times for each combination. The average distribution of IJs in the three Y-tube compartments is presented in Fig. 37. In experiment A, 677.3 ± 35 at 8 °C and 697.3 ± 68 at 15 °C of 1,000 ± 180 IJs were recovered by the Cobb`s sieving method. In experiment B, 780 ± 58.8 IJs at 8 °C and 701.5 ± 64 IJs at 15 °C and experiment C, 705 ± 67.8 and 665.3 ± 71.5 IJs of 1,000 ± 120 IJs were recovered at 8 and 15 °C, respectively. The distribution of IJs was calculated according to the number of recovered IJs and significant differences were recorded (A: $F = 15.0$; $df = 5, 12$; $p = 0.000083$; B: $F = 15.0$; $df = 5, 12$; $p=0.00081$; C: $F = 43.2$; $df = 5, 12$; $p<0.00001$). In general, a high number of EPN stayed at the point of application. This number was highest when only oil seed rape plants were offered (Fig. 37 C). Whenever insects were present, more EPN migrated towards the insect (Fig. 37 A and B). Thus the positive chemotaxis towards oil seed rape roots is much lower than to the insect larvae at the both temperatures. The response of IJs toward the stimuli increased with increasing temperature. While at 8 °C significantly more IJs remained in control, at 15 °C significantly more IJs were found in compartment with *D. radicum*.

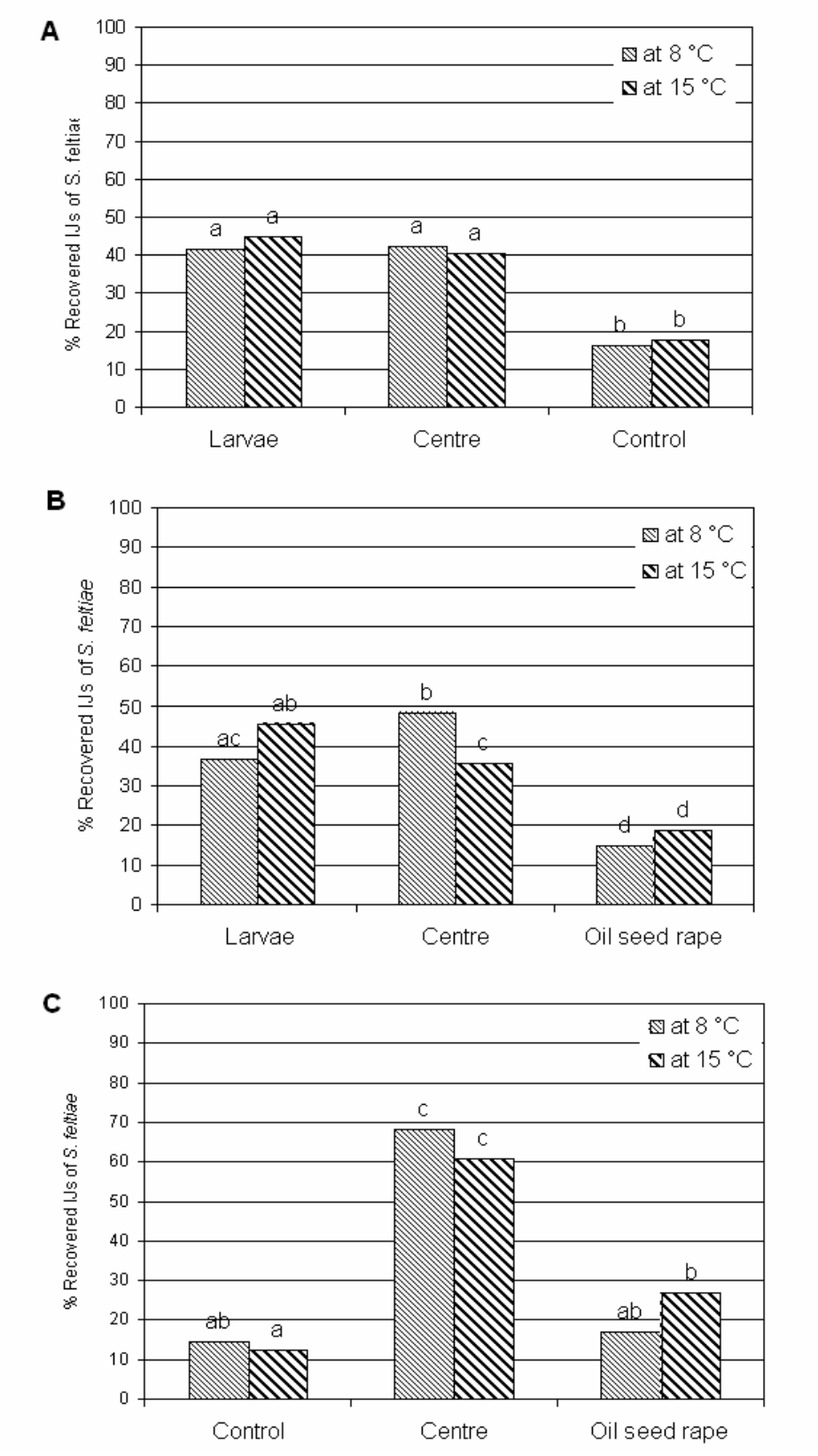

Figure 37. Distributions of IJs of *S. feltiae* washed out of the sand recovered from the different compartments of a Y-Olfactometers (Fig. 5), which had been incubated at 8 and 15 °C for ten days. Nematodes (1,000 per Olfactometer) were inoculated into the center compartment. (A) Five larvae of *D. radicum* in one compartment and no stimulus in the control compartment, (B) Five larvae of *D. radicum* in one compartment and an oil seed rape plant in the other, (C) an oil seed rape plant in one compartment and no stimulus in the control. Columns with the same letter are not significantly different ($P<0.05$).

4. Discussion

Entomopathogenic nematodes (EPNs) are biocontrol agents and have been a focus for research since Glaser demonstrated their potential as control agents of insect pests (Glaser, 1931; 1932). Subsequently, extensive research on the nematode pathogenicity, bacterial symbiont function and insect defence mechanisms was carried out in different laboratories especially in Europe and USA. With the progress in large scale liquid culture production (Ehlers, 2001) the research has expanded in many different directions. EPNs represent an important part of the spectrum of biopesticides. They have been used to control insect pests in high value crops and they could be used in large scale in sustainable agricultural systems.

Although the use of EPNs as biocontrol agents has expanded over the past decades, it is still mainly restricted to high value crops that cover a small proportion of the world agriculture market. Besides economic factors, the restricted expansion of the use of EPNs as biocontrol agents is due to various intrinsic biotic and abiotic related factors. As the interest in the use of indigenous or exotic nematodes is increasing, knowledge on the ecology and specific environmental requirements of each specific species or strains of nematodes that we want to use as a biocontrol agent becomes crucial.

Though much research has been done and much progress has been made, especially in the last decade, the establishment and persistence of infective juveniles (IJs) of EPNs after release in out door environments remains obscure (Boff, 2001).

4.1. Detection of EPNs

In order to detect indigenous EPNs prior to release of *in vitro* produced populations, soil samples were collected and checked for the presence of naturally occurring populations. Sturhan (1996) pointed out that in the upper 15 cm of soil, 56.6 % of the nematodes were present, 40.6 % in the next 15 cm downwards.

The occurrence of EPNs in a given habitat partly reflects the ecology of the nematodes, because occurrence is a sum of parameters including survival, dispersal, host finding and reproduction. Thus, a reliable detection method for the nematodes is a prerequisite for using data from a survey. Several methods for detection are available and these are compared in Table 10. The live bait method is most commonly used. The advantage of this method is that

it is simple and selective towards EPNs. The disadvantage, however, is that it is less quantitative, because it depends on successful infection of the bait larvae. This can be improved by counting the number of invading nematodes by dissection. Baermann extractions and flotation methods are not selective towards EPNs and both, nematological experience and special equipment is required. Although these methods are regarded as quantitative, the extraction efficacy can be various. Examples of this were given by Barbarcheck and Kaya (1991), and Kung et al. (1990a).

Sturhan and Mracek (2000) compared the methods, *G. mellonella* baiting technique and direct extraction method for recovering IJs of *Steinernema* from soil collected in Germany and the Czech Republic. The result of the study show that all *Steinernema* species were recovered with both methods, but the baiting technique was generally less effective and mixtures of species were frequently undetected. The direct extraction method provided quantitative estimates of IJs density, but no information on their infectivity or morphological characters of adult nematodes because cultures cannot be established (nematodes cannot survive the extraction process). Hass et al. (1999) stated further that the most efficient extraction method of IJs from soil was the centrifugal flotation using the Baermann funnel and dissection of bait insect.

Table 10. Comparison of methods used to detect EPNs in soil samples.

Method	Selective towards EPN	Quantitative	Qualitative [a]	Method possible without		References
				special equipment	nematological experience	
Live-bait (*Galleria* trap)	Yes	(yes)[b]	yes	yes	(yes)[c]	1
Dissection of bait insects	Yes	yes	yes	yes	(yes)[c]	2, 3, 4
Baermann extraction	No	yes	yes	no	no	3, 5, 6
Extraction by flotation	No	yes	no	no	no	3, 5, 6

[a] The ability of the nematodes to migrate through the soil (Baermann, bait) and to infect an insect (bait)
[b] Depends on the number of bait larvae used
[c] The selectivity of the method supports identification.
[1] Bedding and Akhurst (1975), [2] Curran and Heng (1992), [3] Bednarek (1998), [4] Koppenhöfer et al. (1998), [5] Saunders and All (1982), [6] Sturhan (1995).

In the presented thesis, the live bait and dissection of bait insects was used in order to detect endemic and to recover inoculated EPNs from release areas. Establishment and persistence of EPNs in field experiments were measured by using of the live bait with last instar larvae of *G. mellonella* and given as the percentage of positive soil samples in which EPN were recorded.

4.2. Natural occurrence of EPNs in Germany and northern Europe

An overview of surveys for EPNs in agricultural soils in Germany and northern Europe is given in Table 11. In agriculture fields, only *Steinernema* species were detected and the most abundant species were *S. feltiae* and *S. affine*. The study of Bednarek (1998) and Sturhan (1996) were based on quantitative methods. Bednarek dissected bait larvae and on that basis estimated a population density of 600-4,300 IJs/m² in the upper 25 cm soil layer at an experimental farm in Poland. Sturhan (1996) stated that *Steinernema* was present in 94 % of the samples; about 97 % of all specimens proved to be *S. affine* and 3 % *S. feltiae*. *Heterorhabditis* species amounted for less than 4 % of all records of the occurrence of EPNs. *H. bacteriophora* was only isolated once from agricultural field in Germany (Sturhan, 1997). In this thesis, only *S. feltiae* species was found in 1.76 % of the soil. In contrast to Sturhan (1996), no *S. affine* was detected, although most records of *S. affine* came from agricultural fields and grassland (pastures). The results of this study are comparable to results reported by Ehlers et al. (1991), who isolated more *S. feltiae* strains than *S. affine* in North Germany between the years 1985 to 1989. The reason why only *S. feltiae* was detected might be, that indigenous *S. affine* did not respond to the bait insect used. While 82 % of the indigenous *S. feltiae* strains were found on the organic farm Lindhof, 18 % were isolated from the conventional farm in Rastorfer Passau (Table 4).

Table 11. *Steinernema* species isolated during surveys of agricultural soils in northern Europe. Steinernematids have also been reported from England[1] (*S. feltiae* and *S.* sp.), Norway[2] (*affine, feltiae, intermedium* and sp.) and Sweden[3] (only *S.* sp.), but it was not possible to conclude from the publications whether the species had been detected in agricultural soils.

	S. affine	*S. feltiae*	*S. bicornutum*	*S. intermedium*	*S.* sp.	References
Belgium	-	-	-	-	-	Miduturi et al., 1996
Denmark	+	+	+	+	-	Nielsen, 2000
England	-	-	-	-	-	Chandler et al., 1997
England/Holland	+	+	-	-	+	Hominick et al., 1995
Finland	-	+	-	-	+	Husberg et al., 1988
Germany (I)	+	+	-	-	-	Ehlers et al., 1991
Germany (II) [a]	+	+	-	-	-	Sturhan, 1996
Ireland	+	+	-	-	-	Griffin et al., 1991
North Ireland	-	+	-	-	-	Blackshaw, 1988
Scotland	-	+	-	-	-	Boag et al., 1992
Poland	-	+	-	-	-	Bednarek, 1998

[1] Hominick and Briscoe, 1990 ; [2] Haukeland, 1993; [3] Burman et al., 1986.
[a] Sampling was restricted to one experimental farm.

4.3. Application of EPNs into soil

EPNs can be applied in suspension (with sprayer, hoses, irrigation pipes, sprinkler system, injection equipment or dipping method of plants). Some attempts have been made to release infected insects. Most often, the nematodes are sprayed with a beam or applied as a drench using large volumes of water to wash them down into the soil (Smits, 1996). A major factor limiting the success of biologicals in the fields is the method by which they are applied; application methods that succeed in greenhouses are often inappropriate in the fields. Since agrochemicals dominate crop protection (more than 98 % of all crop protection products are chemicals), biological products must be applied with conventional application systems. With respect to EPNs, their requirements for high levels of soil moisture necessary to develop their full efficacy can cause problems during field use. Application volumes (litre/ha) in broad hectare crops are normally around 200 l/ha, occasionally as high as 800 l/ha in orchard and vine crops. Application volumes more than 800 l/ha are relatively rare, with exception such as turf in golf courses and irrigated crops (Chapple et al., 1995). *S. feltiae* and *H. bacteriophora* used in this study were applied in volume around 420 l/ha; the application volume per unit area was 0.04 l/m². In general, drop spectra can be largely ignored when applying nematodes. Nematodes are large relative to the drops produced by conventional nozzles. It is generally accepted that drops with a diameter of more than 300 µm have a minor impact on plant surfaces as they either re-distributed by bouncing and shattering off the leaves (Schaefer and Allsop, 1983). Therefore, it may be better to use drops with a diameter >300 µm for soil application and also for applications to the canopy in order to get nematodes onto the soil surface and reach the soil pests. Thus, IJs applied onto crops, can easily move through plant surface towards the soil surface. The drop size in the presented study had an average size of 1,212 ± 648.8 µm (Tab. 6). The optimal IJs dosage is approx. 50 IJs/cm², which can be applied with this drop size, even in relatively high crops. Therefore, inoculated nematodes can establish into soil without any problem by adjusting the drop size. But nevertheless a negative correlation between the height of the crops and the number of IJs reaching the soil surface was recorded (Fig. 21). Besides the amount of nematodes the quality of EPNs plays a key role for their establishment and persistence. Quality was measured by using Petri dishes immediately after application to check whether the application system was suitable for nematodes. According to the results, IJ survival and pathogenicity before and after application was not significantly different. (Tab. 5). The results indicated that the application system used in this study was considerably comfortable for the nematodes. As the equipment is

comparable to conventional sprayers used in agriculture, spraying should not significantly affect EPN quality.

Soil temperature can also affect nematode efficacy. Warmer temperatures reduce nematode survival while cooler temperatures increase activity and infectivity. Soil temperature between 12 to 28 °C is considered favourable for application of most EPNs. If soil temperature is above 28 °C, a pre-application irrigation is usually recommended to reduce soil temperature before nematode application (Grewal et al., 1994a). However, in this study, 20 % of the applications were carried out below 12 °C of soil. The minimum temperature of soil temperatures was 5 °C, but *S. feltiae* and *H. bacteriophora* applied at this temperature persisted for 12 months in these fields, while *H. bacteriophora* sprayed at 17.5 °C persisted for 22 months (Fig. 12). It can thus be concluded that for *H. bacteriophora* application at warmer temperature is more favourable than for *S. feltiae* to achieve for longer persistence.

4.4. Factors with impact on establishment

Factors with influence on EPN during the time of application and over the following few hours are most critical for the establishment and persistence of EPNs in the soil. Ultraviolet radiation can have major effects on EPNs. Gaugler et al. (1992) concluded from their experiments that 60 min of exposure to direct sunlight can inactivate *S. carpocapsae*. *H. bacteriophora* was even more sensitive and was inactivated after 30 min exposure to sunlight. Georgis and Gaugler (1991) pointed out that spray application in the evening or in cloudy weather could help to avoid this problem, but there is also evidence that the time of spraying does not greatly affect nematode survival. Post-application irrigation, to rinse nematodes down, usually improves their persistence (Selvan et al., 1994; Glazer and Navon, 1990; Glazer 1992). In the present study, EPNs were sprayed in the early morning or during cloudy weather (Tab. 7 and 9). The analysis of the results on establishment and climatic conditions indicate a correlation between the amount of weekly precipitation (mm) and the number of positive soil samples immediately after application (Fig. 23, 24 and 25). Optimal precipitation in a week can vary from 15 to 30 mm/week.

EPNs can be affected by dehydration, but are capable of entering into an anhydrobiotic state in which their survival ability is improved (Womersley, 1990). This may explain why the nematodes survive better under dry soil condition than in moist or wet soil as shown by Kung

et al. (1991) with their work with *S. carpocapsae* and *S. glaseri*. Even though the nematodes survived, their pathogenicity could have been affected. However, the pathogenicity was in general inversely correlated to soil moisture after rehydration (Kung et al. 1991).

4.5. Factors with impact on persistence

4.5.1. Impact of antagonists

Nematode antagonists can play a role in the reduction of applied and established EPNs. Nematode trapping fungi, collembolans, predatory mites and predatory nematodes can reduce population of EPNs (Walter, 1987, 1988; Epsky et al. 1988, Stirling, 1988; Kaya, 1990; Kaya and Thurston, 1993; Gilmore and Potter, 1993; Koppenhöfer et al., 1996; Wilson and Gaugler, 2003). Ishibashi and Kondo (1986) also stated that IJs placed in sterilized or pasteurised soils survive longer than IJs placed in untreated soils. It is, however, extremely difficult to quantify the impact of antagonists under natural conditions. The decline they cause in the numbers of nematodes will probably be gradual rather than rapid. To exclude the influence of natural antagonists in laboratory experiments, the soil samples from the field were sterilized. In laboratory studies, no correlation was found between the numbers of recovered IJs of *H. bacteriophora* from sterilised or non-sterilised soil collected from the experimental farms. The soils were incubated at 15 and 25 °C for 9 weeks. No statistically different differences in persistence were detected (Fig. 20). Kondo and Ishibashi (1986) compared recovery of *S. feltiae* and *S. glaseri* from sterilised and non-sterilised soil. After eight weeks the number of nematodes in sterilised soil was almost unchanged while the numbers in un-sterilised soil was approaching zero. Persistence in soil from the Lindhof and Rastorfer Passau, however, was not detected after 5 weeks and an influence of potential antagonists in non-sterilized soil was not found. Whereas the persistence in sterilized soil from the Lindhof was lower at 15°C but not at 25°, differences between sterilized and non-sterilized soil were not recorded in soil from Rastofer Passau. Other than expected, persistence in sterilized soil was even lower than in non-sterilized soil from the Lindhof. One possible reason might be that the sterilized soil contained a high content of organic matter which after sterilization was decomposed by newly introduced or surviving microorganisms (in fact, in some samples fungal growth was detected during the experiments). Their metabolic activity might have had detrimental impacts on EPN persistence. In general, the

persistence is higher at lower temperature and after 5 weeks hardly any nematodes were found at 25°C.

4.5.2. Soil temperature

Environmental conditions can have tremendous influence upon survival rates. Temperatures above 40°C and below 0°C are lethal for most EPN species. Griffin (1993) and Grewal et al. (1994) have reviewed the temperature ranges for survival and reproduction of EPNs. In the range of 15-25°C, higher temperatures increase the rate of metabolism and shorten the life span. The resistance to heat can vary considerably between species. Susurluk et al. (2001) compared the heat resistance of a Turkish strain of *H. bacteriophora* (TUR-H2) and *S. feltiae* (TUR-S3) and the *S. feltiae* strain survived better than *Heterorhabditis* sp. at 32°C. However, the extreme high temperature is of less importance for persistence, whereas temperatures below 0°C have been. Populations persisting for over 24 months had been exposed to temperatures below 0°C for many weeks. Thus laboratory experiments seem to miss a major factor which could explain why they survived in the field, but did not in the laboratory. One reason might be that nematodes have survived in host insects.

The persistence of *S. feltiae* and *H. bacteriophora* at 8 °C in soil without any hosts was compared in laboratory assays over period of 12 weeks. Persistence was measured as number of IJs isolated from baiting insects. The results showed that while the amount of *S. feltiae* hardly changed (approximately 34% recovered) between 4^{th} and 12^{th} weeks (Fig 18), the number *H. bacteriophora* DJs declined until none were isolated anymore after 12 weeks (Fig 19). These results support the assumption be Strong (2002) that steinernematids usually have a longer half-live than heterorhadbditids. Particularly at low temperature *S. feltiae* seems to persist very well as also indicated by the field results obtained in oil seed rape (Fig. 17). The number of positive samples did hardly change over the winter and even increased in one month. Although this record was after a period of higher temperature, one must consider that nematode dispersal must have caused the higher incidence of positive samples. Lower temperature before and after this increase supported survival. If compared to records on *H. bacteriophora*, such high numbers of positive samples were never recorded over a period of 5 months during the winter.

The other persistence experiments with *H. bacteriophora* were carried out at 15 and 25 °C. In this experiment the persistence was even lower. No DJs were recovered after 5 weeks (Fig 20). In conclusion, *S. feltiae* was significantly more enduring than *H. bacteriophora* and also seems to respond better to lower temperature. The results are in agreement with results of Molyneux (1985) and Kaya (1990) who documented that following field application, the persistence of large numbers of *H. bacteriophora* was limited to a period of weeks rather than mouths, whereas *Steinernema* spp. generally persisted for more extended periods. Gaugler (1981) also noted lower survival rates for heterorhabdits than for steinernematids. According to the presented result, in pot experiments the half-life time of *H. bacteriophora* at 8 °C was 24.8 days after application. Similar results are supported by Baur and Kaya (2001) who reported low rates of survival of *Heterorhabditis* sp. with a mean half-life of 34 days. Molyneux (1985) documented that *S. carpocapsae* at 10 °C had an estimated half-live of 330 days. But it declined rapidly to 11 days at 15 °C and to only 5 days at 28°C. These results also indicate that lower temperature supports EPN persistence. However, this conclusion cannot be generalized as the tolerance to low temperature has a limit.

4.5.3. Soil humidity

Dry conditions adversely affect nematode motility and survival. Some free-living stages of several animal and plant parasitic nematodes can survive exposure to desiccation for long period (Cooper and Van Gundy, 1971; Wharton, 1986). The potential of EPN to survive desiccation, however, is poor (Glaser 2002). Surrey and Wharton (1995) tested the desiccation survival of *H. zealandica*. Their experiment indicated that survival was poor once water had been lost from the substrate. Menti et al. (1997) showed that although *H. megidis* survival was superior to that of *S. feltiae*, desiccation tolerance for both species was poor (minutes). Liu and Glazer (2000) stated that desiccation induced only a shallow anhydrobiotic state of heterorhabditids from Israel. Tarasco and Griffin (2003) reported that at 84 % relative humidity *S. carpocapsae* had a desiccation tolerance of at least 5 days with a survival percentage of 30 %, while *S. feltiae* and *H. bacteriophora* survived less well (11-26 % after 24 h) and no living IJs were found after 28 h. Thus desiccation must be considered as limiting factor, but should, under normal agricultural conditions not have a major impact on nematode survival. Simons and Poinar (1973) found that EPN survived low humidity in soils when the pF had reached the permanent wilting points of plants. Such extreme conditions were never reported during the entire period of the experiments.

4.5.4. Soil acidity

The most favourable pH regarding nematode survival was found to be pH 8 for the species *S. carpocapsae* and *S. glaseri* (Kung et al., 1990). This indicates that under normal growing conditions, EPNs would not be negatively affected by pH. There are only few results where nematode occurrence and soil pH was correlated. Miduturi et al. (1996) isolated EPNs within the range of pH 4.0-8.1. Fischer and Führer (1990) documented that pH< 4.0 can adversely affect nematode host finding. However, these conditions certainly have not had any influence on the results recorded within this study.

4.5.5. Soil type

Soil type can directly affect persistence of EPNs. A relatively low number of isolates were found in soil with high clay content in most surveys. Kung et al. (1990) studied the effect soil types on the survival of *S. carpocapsae* and S. *glaseri*. *S. glaseri* survival levels were lower than those of *S. carpocapsae*. Survival decline most rapidly in clay soil followed by clay loam and sand or sandy loam. Rovesti et al. (1991) also studied the persistence of inoculated EPNs in silty clay soil and in clay soil under field conditions. In general, all species used in their study persisted longer in silty clay than in clay soil. Thurston et al. (1994) showed that a high salt content of soil could inhibit the movement and infectivity of EPNs, but did not kill them.

4.5.6. Competition

An indigenous nematode species better adapted to the local climatic conditions and to the potential host theoretically has the potential to impact persistence of a released population. As the risk of replacement is discussed as a possible consequence of an introduction of an exotic species (Barbercheck and Millar, 2000) a failure of establishment or persistence of the introduced species also must be discussed. The indigenous *S. feltiae* and the released *H. bacteriophora* populations were often found at the same time. However, it was detected once that the indigenous species was more frequently isolated after the introduced had disappeared (Fig. 10). The experiments did not yield enough results to draw a final conclusion. Kondo (1989), Alatorre-Rosas and Kaya (1991) and Koppenhöfer et al. (1995) stated that under laboratory conditions two nematodes species may infect the same host individually, but Grewal et al. (1997) and Glazer (1997) pointed out that under natural conditions, dual

infection are unlikely because steinernematids are repelled from infected hosts when infection had happened 6-9 h before. While this avoidance reduces interference, the combination of two nematodes against one target is likely to lead to a competitive exclusion of one species in the long run (Koppenhöfer et al., 1996).

4.5.7. Energy resources

Another important factor on survival of the nematodes in the soil is depleting energy resources. IJs do not feed and so they dependent on internal energy resources, which enable them to survive until a host is located. Quantitative and qualitative analysis of biochemical reserves of EPNs has been conducted by Selvan et al. (1993) and Abu Hatab et al. (1998) showing that lipid content and fatty acid composition are highly dependent on media components. Several studies have shown (Lewis et al., 1995a; Patel et al., 1997; Patel and Wright, 1997) that nematode infectivity declines as energy reserves are depleted. This factor certainly has influenced persistence particularly at periods of high temperature in summer, when energy consumption is higher than at low temperature during winter.

4.5.8. Influence of the crop

Persistence in the different crops was quite variable. While the longest persistence of *H. bacteriophora* was 22 months in beans followed by wheat with an under-sowing of red clover (Fig. 12 and 14), the shortest was only 0 and 1 month in potato field (Fig. 16 and 26). Longer persistence of the nematode was detected in lupine (13 months), oil seed rape (12 months) and wheat (13 months) (Fig. 15 and 16). Although in 2004 the experiments were concentrating on establishment, at least some data on persistence (4 months) were resulting from the investigation. The 2004 results support the conclusion that considerable persistence can be recorded in cereals. In wheat and barley the incidence of positive soil samples even increased (Fig. 26). Oil seed rape supported *S. feltiae*; the longest persistence period was 11 months until the end of the investigation (Fig. 17), and also *H. bacteriophora* (Fig. 26).

Rather surprising are the results recorded from pasture. In the long-term persistence study, *H. bacteriophora* was recorded for 10 months with a sharp decline two months after establishment. After the winter the population did not recover and was not detected again after April (Fig. 12 and 15). When applied in March 2004 the establishment was low (16% positive

soil samples) and in the following months the population was never re-isolated. When applied in April 2004 the establishment was much more successful (77.5% positive samples), but as recorded before, a dramatic decline was recorded in the following month and the population was not detected again in August. The application in June succeeded to establish the nematodes, but they were not detected in the following two months. In conclusion, the persistence of *H. bacteriophora* in pasture is low. Although one should consider that in pasture nematodes are less often disturbed and should find host insects (noctuids, elaterids, curculionids), the presented results indicate that persistence is low. Forschler and Gardner (1991) reported persistence of *H. bacteriophora* for 5-8 weeks in pasture. Klein and Georges (1992) found high levels of parasitism one year after applying *H. bacteriophorea* and *S. carpocapsae* to turf grass. Sulistiyanto and Ehlers (1996) recorded persistence of *Heterorhabditis megidis* and *H. bacteriophora* in turf grass for 309 and 265 days, respectively. *H. bacteriophora* has been applied on golf courses in Germany to control grubs of *Phyllopertha horticola* since 1997. The results of surveys between 1999 and 2002 indicated that in 1999, at least one soil sample of 80 % of the golf courses contained *H. bacteriophora*, whereas all controls were negative (Ehlers et al., 2003). In 2002, at least 20 soil samples per fairway were taken from 13 different golf courses, which had been treated the years before. Of 712 samples, 19 % contained *H. bacteriophora* and 47 % of the fairways were positive. From half of the golf courses, which had used the nematode in 1998, the nematodes were re-isolated, from 75 % of those that had been treated in 1999, from 18 % of those that had been treated in 2000 and from 57 % of those treated in 2001. The low establishment and persistence in samples taken from locations which had been treated in the year 2000 was probably related with low humidity in August in that year, the major period of application. All these results indicate that *H. bacteriophora* can well persist in turf, which is similar to pasture. What can have caused the disappointing results obtained in this study in pasture? The difference between the studies, which report long term persistence, in turf in Germany for more than 4 years, and the present study is, that the reports on long term persistence have always been related with applications against grubs (Coleoptera: Scarabaeidae), whereas no grubs were found on pasture. Thus we can conclude that the presence of hosts has a major impact on persistence.

4.5.9. Availability of hosts

The major factor on long term persistence of EPNs is the presence of host insects and thus indirectly the host plant, providing a basis for the insect population. Soil temperature, humidity, acidity and soil type thus must be considered of minor importance and their impact is probably only then of major importance on survival, when extremes are reached which can dramatically reduce an EPN population. However, all these factors have a more immediate impact on host finding. The presence of a suitable host within travelling distance is a critical parameter for the survival of a nematode population. The longest distance nematodes have been reported to travel in laboratory experiments are in the magnitude of decimetres. But even within decimetres, their host finding capacity is heavily influenced by the characteristics of the soil, the crop and the host. As *H. bacteriophora* cannot persist for much longer than a month even at lower temperature in the absence of hosts (laboratory persistence), the persistence in the field can only have been caused by offspring recycling in an available insect population. This assumption is strongly supported by the fact that in the field in which the maximum persistence of 23 months was recorded, a considerable number of *S. lineatus* was encountered (Fig. 14). Red clover was cultured on this field with winter wheat as cover crop also providing good conditions for the curculionid population. The extraordinarily high number of positive samples of a released *S. feltiae* population was also correlated with the occurrence of a potential host insect (*D. radicum*). Results reported by Strong (2002) on food webs in a bush lupine ecosystem with the lepidopteran species *Hepialus californicus* and *H. marelatus* as a naturally occurring antagonist show a strong relation between the availability of host insects and the nematode population. Sulistiyanto and Ehlers (1996) sprayed *Heterorhabditis megidis* and *H. bacteriophora* on turf. Both species persisted due to recycling in the grubs *Aphodius contaminatus* and than latter controlled *Phyllopertha horticola* and again *A. contaminatus* in the following year, 309 days after the first release. It is a reasonable and trivial conclusion, but how to explain the persistence of *H. bacteriophora* for one year in winter wheat followed by barley, which soil inhabiting host insects are found in these crops? One might consider that they could have survived on elaterid hosts, which are more common in the organic rotation system. However, these records were made on the conventionally managed farm (Fig. 15). Although the necessity for the presence of potential host insects in obvious, the lack of potential hosts in cereal remains to be explained.

4.5.10. Agricultural practices

The establishment and persistence experiment were conducted in organic and conventional farms simultaneously. According to the results, 18 % of the indigenous records of *S. feltiae* were from the organic farm Lindhof. Thus many more were isolated from the conventional farm in Rastorfer Passau (Table 4). However, differences in the establishment and persistence of released *H. bacteriophora* between both locations were not detected. Prolonged (10-20 days) exposure to high concentrations of inorganic fertilizers inhibits EPN infectivity and reproduction, whereas short (1 day) exposures increased infectivity (Bednarek and Gaugler, 1997). Sturhan (1996) found no such correlation. Pesticides must be excluded as influencing factors. Most insecticides do not interact with EPN (Zimmerman and Cranshaw, 1990; Ishibashi and Takii, 1993). The opposite was documented by Koppenhöfer et al. (2000) who recorded synergistic effects *Heterorhabditis* spp. and *S. glaseri* and the neonicotinoid imidacloprid and the chemical did not compromise nematode recycling in grubs (Koppenhöfer et al., 2003).

A major impact on persistence seems to be related to tillage. The extremely short persistence of only one month in a potato field (Fig 16) might have been caused by earthing up of the plants. After intensive tillage in potato field, no more positive soil sample contained *H. bacteriophora* although enough IJs (42.6 ± 5,3 IJs/cm²) had reached the soil surface. However, tillage with disc harrow and plough seem to have little effects on persistence of *H. bacteriophora* (Fig. 13, 15 and 16). Tillage in the field with 23 months persistence, on the other hand, seemed to have reduced the incidence of positive samples (Fig. 14). Lack of physical disturbance and favourable soil conditions favours the success of control attempts using EPNs (Shapiro-Ilan et al., 2002). Under a conventional tillage regime, the soil surface tends to have greater fluctuations in temperature and moisture than under no-tillage or reduced tillage management and EPNs are often more frequently detected in reduced tillage regimes (Brust, 1991; Hsiao and All, 1998; Hummel et al., 2002; Millar and Barbercheck, 2002; Shapiro et al., 1996). Brust (1991) reported that no tillage and the presence of weeds increased infection of *G. mellonella* caused by EPNs in the first and subsequent years. This was probably because of the presence of ground cover, which helped to keep the soil moist compared with the bare soil surface of the conventional tillage treatment. Brust (1991) also studied the influence of tillage, weed and irrigation in a cropping system with corn. Irrigation had no influence on the nematode population (*H. bacteriophora*) whereas tillage reduced the

population size and persistence of weed increased the population size. More detailed experiments should be conduced to study the effect of tillage on persistence.

4.6. Seasonal population dynamics of EPNs

The relation between insects and nematode population thus is well established. It should then not be surprising to find seasonal differences in the occurrence of EPNs. Insect populations entering the soil for pupation will be affected by EPNs, depending on the initial population size of the EPNs. Short term fluctuation of the incidences of re-isolation was observed during the whole study but variation in population dynamics of *S. feltiae* and *H. bacteriophora* were recorded also over the seasons. Like seasonal oscillations the short-term changes are probably a result of variable availability of potential host insects. The results indicated (Fig 15 and 16) that fewer nematodes are recorded during December to March (0 to 8 % positive samples). After March, the number of positive soil samples usually raises again to up to 40 % (Fig 14). Puza and Mracek (2004) studied seasonal dynamics of indigenous EPNs in meadow and oak forest habitats and recorded high nematode densities at the beginning of the season followed by a rapid decrease and stabilisation during the winter. They also pointed out that nematode abundance was significantly correlated with the abundance of suitable insect hosts. Low nematode abundance during winter and the discrepancy between the high spring and low autumn nematode abundance may be explained by the possibility of nematode overwintering in insect bodies. Blackshaw (1988) and Griffin et al. (1991) noted that nematodes were more likely to be found in autumn and winter than spring. Most nematodes were found in samples taken in July to October and the lowest number of positive soil samples was recorded in May. Insect population build up during summer and as a result, nematode population increase in late summer and in autumn. The spatial distribution of the nematodes in the soil is an important part of their population dynamics. It has been demonstrated that the distribution of nematodes is uneven and patchy (Cabanillas and Raulston, 1994; Stuart and Gaugler, 1994; Sturhan, 1996; Nielsen, 2000).

4.7. Genetic stability and adaptation

Significant differences were recorded between laboratory populations and re-isolated populations in infectivity, reproduction potential and persistence. It might be concluded that the laboratory population has genetically deteriorated. However, the origin of the re-isolated

population is the laboratory population. If the laboratory generation had decreased in its control potential then this change has happened within the year following the release of the re-isolated population. Another possibility is that the higher fitness of the re-isolated population is a result of selection. Johnigk et al. (2002) and Strauch et al. (2004) demonstrated that beneficial trait of the strain used in this study can be improved by genetic selection. The laboratory strain was not exposed to environmental stress, whereas the released population had to adapt to natural conditions. If this is what happened with the released population, this is rather good news, as it indicated that the genetic plasticity is sensible to changing selective factors.

4.8. Persistence and efficacy of *H. bacteriophora* against *O. sulcatus*

In order to control larvae of the black vine weevil *O. sulcatus* in plantation, which are using plastic mulch nematodes, EPN cannot be applied to the soil surface. It was therefore considered to apply EPN by dipping roots into a nematode suspension. The nematodes then remain on the surface of the roots by the help of a sticker (0.5% Carboxymethylcellulose). However, if applied during transplanting, nematodes must at least persist for two months until the weevils have laid ages and the larvae have hatched. In potting soil *H. bacteriophora* persisted for 12 weeks maintaining its pathogenicity. Pathogenicity had been measured by the mortality of *T. molitor* larvae added to soil samples. The mortality was rather low at 10% after 12 weeks. The greatest decline was recorded shortly after nematodes had been applied. One day after planting 80% mortality had been recorded and 2 weeks later it had dropped to 25%. The presence of *O. sulcatus* significantly increased the persistence and thus the mortality of *T. molitor* larvae. If larvae were present in the strawberry pots plant, nematodes thus persisted at considerably higher rate than without any larvae (Fig. 33). The results (Fig. 30) indicate that the dipping might be a useful alternative application method to control black vine weevil larvae and plants were protected from attack (Fig. 32). Since the IJs applied by dipping remain in the surrounding of the roots, IJs might even be applied in lower concentration compared to area application of 50 IJs per cm² but this remains to be proven experimentally. Peters et al. (2002) treated strawberry plants by dipping and planted in the field. The trial results indicated that *H. bacteriophora* persisted in the soil for over a year. Mortality of *T. molitor* larvae was up to 95 % until 8 weeks after planting. The mean mortality dropped to 60 % after the winter had passed, 345 days after planting. According to these results, dipping

plant roots into the nematode suspension seems to be feasible to control *O. sulcatus* in strawberry or ornamentals.

4.9. Persistence and efficacy of *Steinernema feltiae* against *Delia radicum*

Third instar *D. radicum* are excellent targets for *S. feltiae*, as has been shown in this study and also by Sulistyanto et al. (1994). The nematodes are attracted by the insects more than they are to oil seed plants, their application resulted in more than 80% control and the nematodes persisted for more than 12 weeks. In the orientation experiments using Y-olfactometers, *S. feltiae* infected 100 % of larvae at the both temperatures when no plants were present in the other compartment. Infection rate was reduced to 60 and 80 % at 8 °C and 15 °C, respectively, when roots were offered as well. The higher temperature increased behavioural response of the nematode towards larvae and roots but there were no significant differences between the two temperatures. Most of the nematodes in these assays stayed at the point of application. The same was observed by Boff et al. (2002) who were working with *H. megidis* and strawberry plants. When *O. sulcatus* larvae and strawberry plant roots were placed in each compartment, more nematodes (41 %) moved towards the arm with the plant roots than towards the host larvae. A likely candidate for attraction is of course CO_2, a general kairomone produced as an end product of the metabolism of plants, microorganisms and other soil animals. CO_2 has been shown to be involved in the long distance attraction of plant parasitic nematodes (Klinger, 1965; Robinson, 1995) and it also attracts EPNs (Gaugler et al., 1980; Lewis et al., 1993; Robinson, 1995). Mc Call et al. (1993) and Dicke (1999) reported that damage caused by insect on the aerial part of the plants could induce the production of secondary metabolites that attract enemies of insect pests. Similar results have been documented by Boff et al. (2002) that larval damage to roots of strawberry and *Thuja occidentalis* obviously stimulated EPNs to migrate to the site where the insect damaged the plant.

Chen and Moens (2003) reported also that four days after application 45% mortality of *D. radicum* was caused by *S. feltiae* (10 IJs/larva). Field trials against *D. radicum* have had variable results (Hommes, 1988; Bracken, 1990; Simser, 1992; Vänninen et al., 1992; Schröder et al., 1996). Nielsen and Philipsen (2004) also reported from field trial that *D. radicum* populations could be suppressed by *S. feltiae* and the number of *D. radicum* puparia was significantly reduced at cabbage plants where *S. feltiae* had been applied in the early

season. Nielson (2003) documented 77 % mortality of third instar larvae of *D. radicum* caused by *S. feltiae*. All these results indicate that control of cabbage root flies in oil seed rape might be a possible strategy. However, at current costs it is not economic to use EPN against this pest. However, if nematodes were able to establish and persist during the following crops (wheat and barley) the application of EPN as an inoculative application should be more economic. The introduction of EPN in the field was less successful when nematodes were applied in spring and early summer. At that time the host density might be too low, as the field had been sprayed with insecticides (Fig. 26). In contrast, the application into the crop in autumn, when cabbage root fly larvae were present, was a full success. Considering that the potential of wheat and barley to serve as crops to maintain a nematode population was quite high, the feasibility of this approach is worth testing at the field level, particularly as problems seem to increase in oil seed rape and chemical measures have only a low potential. Further studies are therefore needed to test this possibility.

5. Summary

Entomopathogenic nematodes (EPNs) in the families Heterorhabditidae and Steinernematidae have considerable potential as biological control agents of soil-inhabiting insect pests. One of the most important factors for sustainable biological control is their successful establishment in the soil and persistence in the field. Objective of this study was to investigate establishment and persistence of *Steinernema feltiae* and *Heterorhabditis bacteriophora* in different field crops and rotation regimes. Nematodes were applied in the field at different times of the year and re-isolated by baiting soil samples with highly susceptible larvae of *Galleria mellonella*. A total number of 10,980 soil samples was collected and investigated for the presence of EPN over the period 2001 until 2004. In 1.8% of the samples from untreated fields indigenous populations of *S. feltiae* were detected. The results on establishment and persistence gave no indication that the indigenous populations were replaced by the introduction of *H. bacteriophora*. Application of EPNs with conventional spraying technique had no negative impact on survival or performance of the nematodes. The height of the plants was negatively correlated with the amount of nematodes reaching the soil. However, plant height had no impact on establishment. Most successful was establishment of *H. bacteriophora* in June. Compared to results recorded during April until July, the establishment correlated with the amount of precipitation in the week following EPN release. In potatoes and pasture establishment of *H. bacteriophora* often failed, whereas this species established well in beans, oil seed rape, wheat and barley. The percentage of nematode-positive soil samples out of 50 per field was usually between 50 and 100% in the first month after application. It dropped considerably after tillage (probably the reason for low establishment in potatoes) and during the winter. In field crops EPN usually persisted not much longer than one year. The longest persistence of *H. bacteriophora* was detected 23 months after release in beans followed by wheat as cover crop over red clover and pasture. In this field larvae of the pea weevil *Sitona lineatus* (Coleoptera: Curculionidae) were detected in soil samples and found infected with the released nematode population. In the laboratory the field soils were tested for persistence of *H. bacteriophora* with and without host insects at different temperature. Host insects always supported persistence and a negative correlation was detected between the soil temperature and persistence. In the absence of host insects, persistence of *H. bacteriophora* was usually not longer than 5 weeks, but reached 10 weeks in one experiment. A half-life of 24.8 days was calculated at 8°C. Persistence of *H. bacteriophora* was high in pots containing larvae of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) on

strawberries. Mortality of the insect surpassed 90% although nematodes had been applied by dipping strawberry roots a months before eggs were laid into the pots. When compared to an *in vitro* reproduced population, *H. bacteriophora* re-isolated from the field one year after release, infectivity, *in vivo* reproduction potential and persistence were significantly higher. *S. feltiae* persisted at high numbers for longer than 12 weeks in laboratory assays without hosts, why no half-life could be calculated. Released in November on a field infested with cabbage root fly larvae *Delia radicum* (Diptera: Anthonomyiidae), *S. feltiae* was recorded in 100% of the soil samples in February and in 80 % of the soil samples after 9 months when the monitoring stopped. In tests in the laboratory > 80% control was achieved against last instars of *D. radicum*. The comparison on persistence in the absence of host insects with the results obtained in the field strongly indicates that persistence is influenced by the presence of suitable host insects and indirectly by the crop. Thus the antagonistic potential of EPN populations can be preserved if host insects are provided by managing a suitable crop rotation regime.

6. Zusammenfassung

Entomopathogene Nematode (EPN) der Gattungen *Heterorhabditis* und *Steinernema* werden mit großem Erfolg zur Bekämpfung bodenbewohnender Insekten eingesetzt. Sollen nachhaltige Effekte im Feld erzielt werden, muss es gelingen, die Nematoden erfolgreich anzusiedeln und ihre Persistenz zu sichern. Ziel der Untersuchung war es, die Ansiedlung und Persistenz der Nematoden *S. feltiae* und *H. bacteriophora* in verschiedenen Feldfrüchten und Fruchtfolgen zu untersuchen. Die Nematoden wurden zu unterschiedlichen Jahreszeiten appliziert und mittels des hoch anfälligen Insekts *Galleria mellonella* aus Bodenproben geködert. In den Jahren 2001 bis 2004 wurden insgesamt 10.980 Bodenproben untersucht. In 1,8% der Proben von unbehandelten Flächen wurden indigene Populationen von *S. feltiae* nachgewiesen. Hinweise über eine Verdrängung der indigenen Art durch *H. bacteriophora* gab es nicht. Die Ausbringung mit konventioneller Applikationstechnik hatte keinen negativen Einfluss auf das Überleben oder die Qualität der Nematoden. Die Höhe der Pflanzen war negativ korreliert mit der Anzahl Nematoden, die den Boden erreichten. Es gab jedoch keine Hinweise dafür, dass die Höhe der Pflanzen eine erfolgreiche Ansiedlung verhindert hätten. Die Ansiedlung war am erfolgreichsten im Monat Juni. Ein Vergleich mit den im Zeitraum zwischen April bis Juli erzielten Ergebnissen ergab eine deutliche Korrelation des Erfolgs der Ansiedlung mit der Niederschlagsmenge in der Folgewoche. In Kartoffeln und auf Weiden versagte die Ansiedlung von *H. bacteriophora* am häufigsten. Erfolgreich war sie dagegen in Ackerbohne, Raps, Weizen und Gerste. Der Anteil positiver Bodenproben lag bei 50 Proben pro Feld und Probenahmezeitpunkt in der Regel bei 50 bis 100 % im ersten Monat nach Ausbringung. Dieser Anteil fiel erheblich ab nach Bodenbearbeitung (wahrscheinlicher Grund für die schlechte Ansiedlung in Kartoffeln) und während der Wintermonate. In Feldkulturen wurden die ausgebrachten Populationen normalerweise nicht länger als ein Jahr nachgewiesen. Am längsten wurde *H. bacteriophora* über einen Zeitraum von 23 Monaten in der Fruchtfolge Ackerbohne, Weizen mit Rotklee-Untersaat und Weide nachgewiesen. In diesem Feld wurden Larven des Blattrandkäfers *Sitona lineatus* (Coleoptera: Curculionidae) in Proben nachgewiesen, die mit der applizierten Nematodenart infiziert waren. Die Persistenz von *H. bacteriophora* wurde mit und ohne Wirtsinsekten im Labor bei unterschiedlichen Temperaturen getestet. Die Anwesenheit von Wirtsinsekten wirkte sich grundsätzlich förderlich auf die Persistenz aus und eine negative Korrelation wurde festgestellt zwischen der Persistenz und der Bodentemperatur. Ohne Wirtinsekten war die Persistenz normalerweise nicht länger als 5 Wochen und nur in einem

Fall erreichte sie 10 Wochen. Eine Halbwertszeit von 24,8 Tagen wurde bei 8°C errechnet. Sofern sich Larven des Gefurchten Dickmaulrüßlers *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) in Töpfen mit Erdbeeren befanden, war eine erheblich längere Persistenz festzustellen und der Wirkungsgrad überstieg 90%, obwohl die Nematoden durch Tauchen der Wurzeln vor der Pflanzung aufgebracht worden waren und einen Monat bevor den Töpfen Insekteneier zugegeben wurde. Eine nach einem Jahr aus dem Freiland re-isolierte *H. bacteriophora* Population war im Vergleich mit einer *in vitro*-produzierten virulenter, hatte ein höheres *in vivo* Vermehrungspotential und überdauerte länger. *S. feltiae* überdauert länger, so dass im Laborversuch keine Halbwertszeit berechnet werden konnte, da die Nematoden in hoher Anzahl über den gesamten Versuchzeitraum von 12 Wochen überdauerten. Im November in einem Rapsfeld mit Befall der Kohlfliege *Delia radicum* (Diptera: Anthonomyiidae) ausgebracht, wurde im Februar in allen Bodenproben Nematoden nachgewiesen und in 80% der Proben zum Ende des Untersuchungszeitraums nach 9 Monaten. Im Labor wurde ein Wirkungsgrad gegen die Larven der Kohlfliege von über 80% erzielt. Der Vergleich der Persistenz in Abwesenheit von Wirtsinsekten, mit den Ergebnissen der Feldversuche lassen den eindeutigen Schluss zu, dass die Persistenz der Nematoden wesentlich von dem Vorkommen geeigneter Wirtsinsekten abhängt und damit indirekt von der Kulturpflanze. Insofern kann das antagonistische Potential einer Nematodenpopulation erhalten werden, sofern die Fruchtfolge geeignete Wirtsinsekten zur Verfügung hält.

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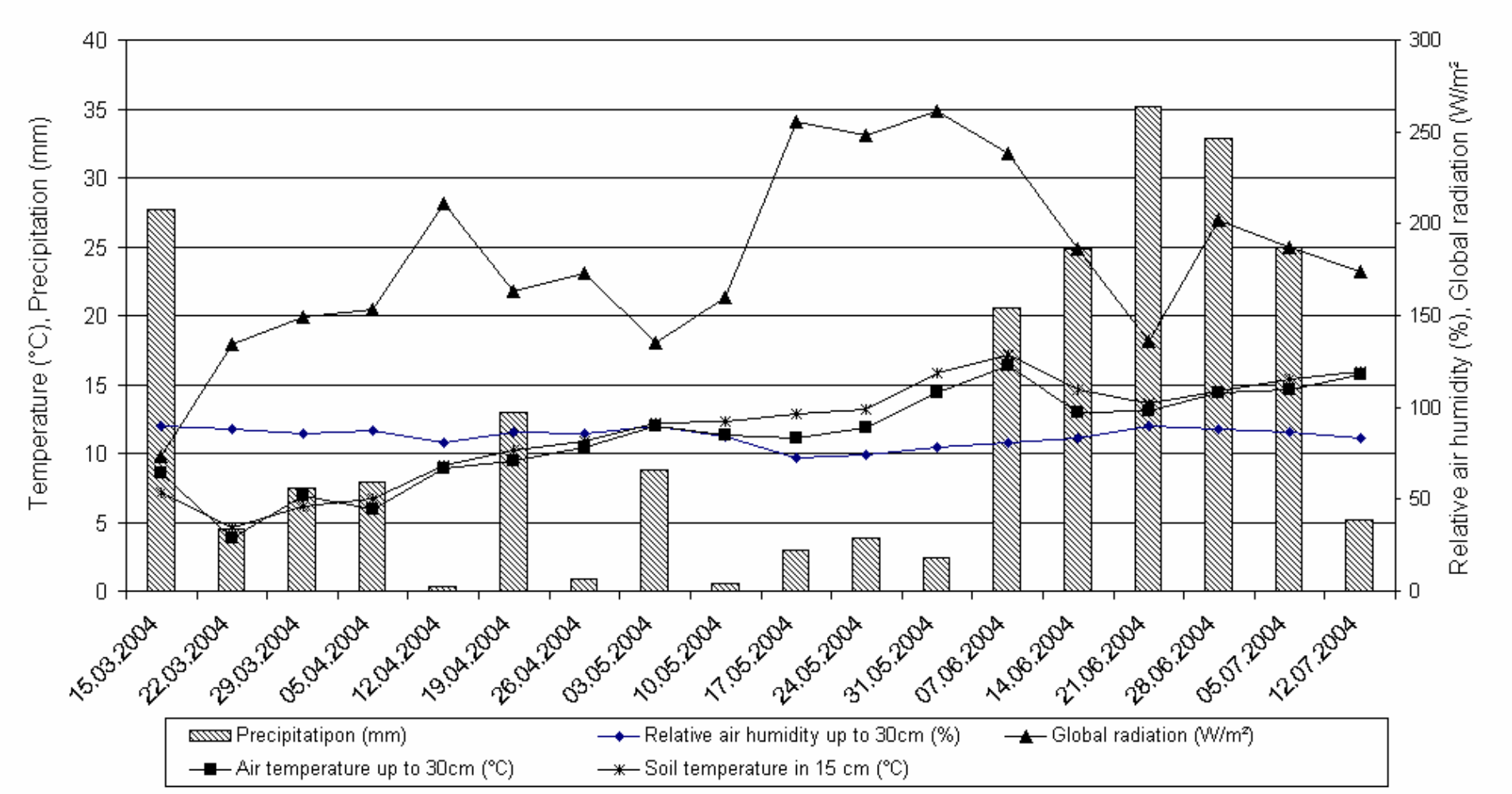
Annex I

Marked numbers indicate significant different of the pairs of means for p=0.05.

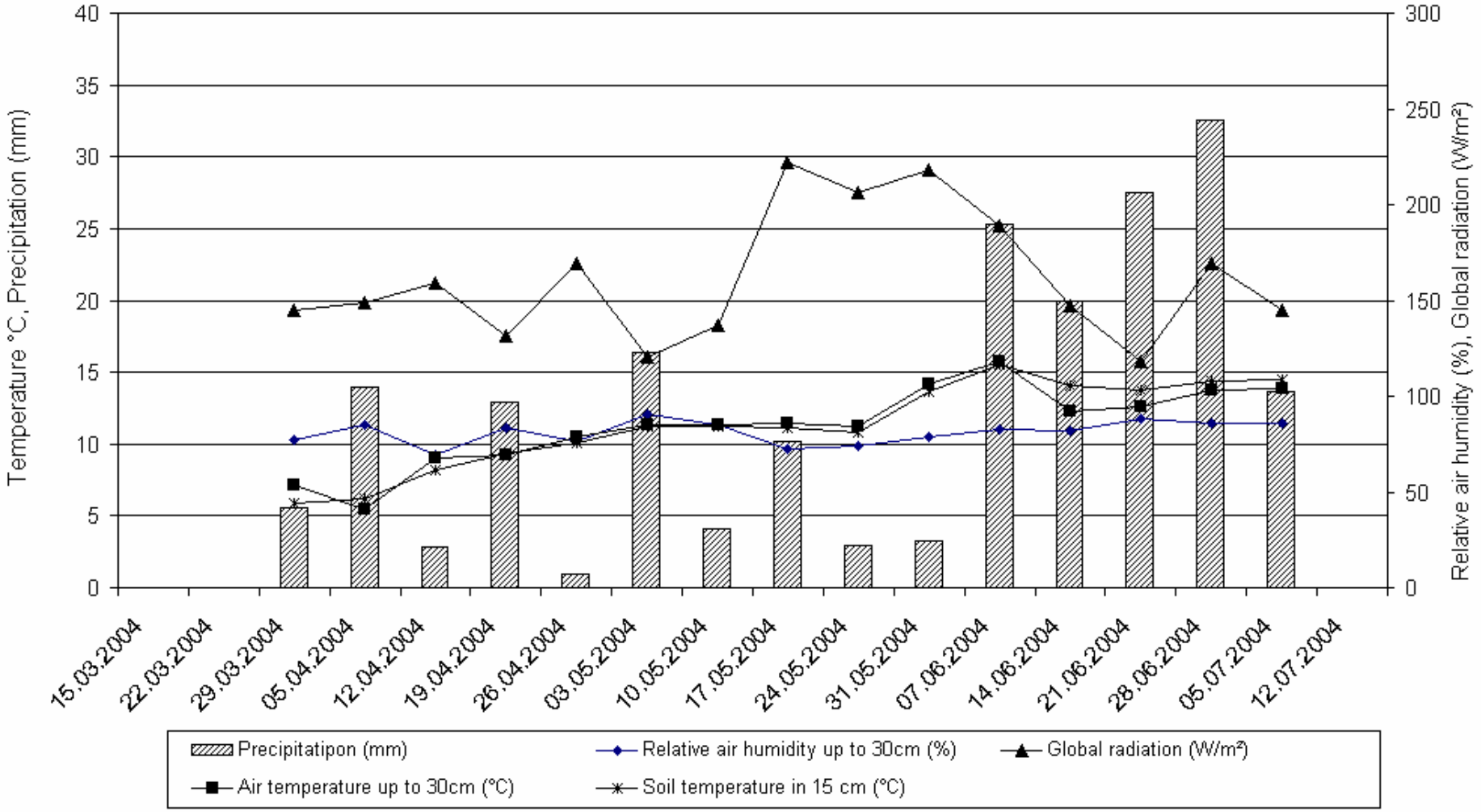
imen		LiHs15 1w	LiHs15 3w	LiHs15 5w	LiHs15 App	LiHs25 1w	LiHs25 3w	LiHs25 5w	LiHs25 App	LiHu15 1w	LiHu15 3w	LiHu15 5w	LiHu15 App	LiHu25 1w	LiHu25 3w	LiHu25 5w	LiHu25 App	RaPas15 1w	RaPas15 3w	RaPas15 5w	RaPas15 App	RaPas25 1w	RaPas25 3w	RaPas25 5w	RaPas25 App	RaPau15 1w	RaPau15 3w	RaPau15 5w	RaPau15 App	RaPau25 1w	RaPau25 3w	RaPau25 5w	RaPau25 App
		90,50	49,75	42,50	169,67	52,75	30,42	25,00	160,83	165,42	165,75	167,42	152,00	65,92	61,25	35,83	123,08	108,50	101,58	54,00	164,17	70,67	46,08	30,42	129,42	138,67	117,17	118,67	154,92	79,67	39,42	30,42	146,17
LiHs15 1w	90,50		40,75	48,00	79,17	37,75	60,08	65,50	70,33	74,92	75,25	76,92	61,50	24,58	29,25	54,67	32,58	18,00	11,08	36,50	73,67	19,83	44,42	60,08	38,92	48,17	26,67	28,17	64,42	10,83	51,08	60,08	55,67
LiHs15 3w	49,75			7,25	119,92	3,00	19,33	24,75	111,08	115,67	116,00	117,67	102,25	16,17	11,50	13,92	73,33	58,75	51,83	4,25	114,42	20,92	3,67	19,33	79,67	88,92	67,42	68,92	105,17	29,92	10,33	19,33	96,42
LiHs15 5w	42,50				127,17	10,25	12,08	17,50	118,33	122,92	123,25	124,92	109,50	23,42	18,75	6,67	80,58	66,00	59,08	11,50	121,67	28,17	3,58	12,08	86,92	96,17	74,67	76,17	112,42	37,17	3,08	12,08	103,67
LiHs15 App	169,67					116,92	139,25	144,67	8,83	4,25	3,92	2,25	17,67	103,75	108,42	133,83	46,59	61,17	68,09	115,67	5,50	99,00	123,59	139,25	40,25	31,00	52,50	51,00	14,75	90,00	130,25	139,25	23,50
LiHs25 1w	52,75						22,33	27,75	108,08	112,67	113,00	114,67	99,25	13,17	8,50	16,92	70,33	55,75	48,83	1,25	111,42	17,92	6,67	22,33	76,67	85,92	64,42	65,92	102,17	26,92	13,33	22,33	93,42
LiHs25 3w	30,42							5,42	130,42	135,00	135,33	137,00	121,58	35,50	30,83	5,42	92,66	78,08	71,16	23,58	133,75	40,25	15,66	0,00	99,00	108,25	86,75	88,25	124,50	49,25	9,00	0,00	115,75
LiHs25 5w	25,00								135,83	140,42	140,75	142,42	127,00	40,92	36,25	10,83	98,08	83,50	76,58	29,00	139,17	45,67	21,08	5,42	104,42	113,67	92,17	93,67	129,92	54,67	14,42	5,42	121,17
LiHs25 App	160,83									4,58	4,92	6,58	8,83	94,91	99,58	125,00	37,75	52,33	59,25	106,83	3,33	90,16	114,75	130,42	31,42	22,17	43,66	42,16	5,92	81,16	121,41	130,42	14,66
LiHu15 1w	165,42										0,33	2,00	13,42	99,50	104,17	129,58	42,34	56,92	63,84	111,42	1,25	94,75	119,34	135,00	36,00	26,75	48,25	46,75	10,50	85,75	126,00	135,00	19,25
LiHu15 3w	165,75											1,67	13,75	99,83	104,50	129,92	42,67	57,25	64,17	111,75	1,58	95,08	119,67	135,33	36,33	27,08	48,58	47,08	10,83	86,08	126,33	135,33	19,58
LiHu15 5w	167,42												15,42	101,50	106,17	131,58	44,34	58,92	65,84	113,42	3,25	96,75	121,34	137,00	38,00	28,75	50,25	48,75	12,50	87,75	128,00	137,00	21,25
LiHu15 App	152,00													86,08	90,75	116,17	28,92	43,50	50,42	98,00	12,17	81,33	105,92	121,58	22,58	13,33	34,83	33,33	2,92	72,33	112,58	121,58	5,83
LiHu25 1w	65,92														4,67	30,09	57,16	42,58	35,66	11,92	98,25	4,75	19,84	35,50	63,50	72,75	51,25	52,75	89,00	13,75	26,50	35,50	80,25
LiHu25 3w	61,25															25,42	61,83	47,25	40,33	7,25	102,92	9,42	15,17	30,83	68,17	77,42	55,92	57,42	93,67	18,42	21,83	30,83	84,92
LiHu25 5w	35,83																87,25	72,67	65,75	18,17	128,33	34,84	10,25	5,42	93,58	102,83	81,34	82,84	119,08	43,84	3,59	5,42	110,34
LiHu25 App	123,08																	14,58	21,50	69,08	41,09	52,41	77,00	92,66	6,34	15,59	5,91	4,41	31,84	43,41	83,66	92,66	23,09
RaPas15 1w	108,50																		6,92	54,50	55,67	37,83	62,42	78,08	20,92	30,17	8,67	10,17	46,42	28,83	69,08	78,08	37,67
RaPas15 3w	101,58																			47,58	62,59	30,91	55,50	71,16	27,84	37,09	15,59	17,09	53,34	21,91	62,16	71,16	44,59
RaPas15 5w	54,00																				110,17	16,67	7,92	23,58	75,42	84,67	63,17	64,67	100,92	25,67	14,58	23,58	92,17
RaPas15 App	164,17																					93,50	118,09	133,75	34,75	25,50	47,00	45,50	9,25	84,50	124,75	133,75	18,00
RaPas25 1w	70,67																						24,59	40,25	58,75	68,00	46,50	48,00	84,25	9,00	31,25	40,25	75,50
RaPas25 3w	46,08																							15,66	83,34	92,59	71,09	72,59	108,84	33,59	6,66	15,66	100,09
RaPas25 5w	30,42																								99,00	108,25	86,75	88,25	124,50	49,25	9,00	0,00	115,75
RaPas25 App	129,42																									9,25	12,25	10,75	25,50	49,75	90,00	99,00	16,75
RaPau15 1w	138,67																										21,50	20,00	16,25	59,00	99,25	108,25	7,50
RaPau15 3w	117,17																											1,50	37,75	37,50	77,75	86,75	29,00
RaPau15 5w	118,67																												36,25	39,00	79,25	88,25	27,50
RaPau15 App	154,92																													75,25	115,50	124,50	8,75
RaPau25 1w	79,67																														40,25	49,25	66,50
RaPau25 3w	39,42																															9,00	106,75
RaPau25 5w	30,42																																115,75

Annex II



Meteorological data in Birkmoor where 10 km far from organic farm Lindhof.

Annex III



Meteorological data in conventional farm Rastorfer Passau.

List of Figures **Pages**

Figure 37. Distributions of IJs of *S. feltiae* washed out of the sand recovered from the different compartments of a Y-Olfactometers (Fig. 5), which had been incubated at 8 and 15 °C for ten days. Nematodes (1,000 per Olfactometer) were inoculated into the center compartment. (A) Five larvae of *D. radicum* in one compartment and no stimulus in the control compartment, (B) Five larvae of *D. radicum* in one compartment and an oil seed rape plant in the other, (C) an oil seed rape plant in one compartment and no stimulus in the control. Columns with the same letter are not significantly different ($P<0.05$). 60

List of Tables **Pages**

Curriculum vitae

Surname: Susurluk

Name: Ismail Alper

Sex: Male

Date of the birth: 19.04.1972

Place of birth: Trabzon, Turkey

Nationality: Turkish

Education

1994-1997 M.Sc, Ankara University, Faculty of Agriculture, Department of Plant Protection in Nematology-Entomology in Ankara-Turkey.

1990-1994 B.Sc, Ankara University, Faculty of Agriculture, Department of Plant Protection in Ankara-Turkey.

1986-1989 Kabatas Erkek Lisesi, High School in Istanbul-Turkey.

1983-1986 Suluova Lisesi, Middle School in Suluova, Amasya-Turkey.

Professional Experiences

1994-2001 Research and teaching assistant in Department of Plant Protection-Entomology, Faculty of Agriculture, University of Ankara-Turkey.

May-October 1999 Short-term scientific visit to Christian-Albrechts-University, Institute for Phytopathology, Department of Biotechnology and Biological Control in Raisdorf-Kiel, Germany, financially supported by NATO scholarship.

July-August 1993 Practical training in the company, Kleinwanzlebener Saatzucht AG (KWS) in Einbeck-Deutschland.

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